A novel approach to latent fingermark detection using aptamer-based reagents

by

Michael Wood

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Certificate of authorship and originality

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of the requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all the information sources and literature used are indicated in the thesis.

Michael Wood

DATE

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Abbreviations

ACE-V	Analysis, Comparison, Evaluation - Verification
ADP	Adenosine diphosphate
AIDS	Acquired immune deficiency syndrome
ALISA	Aptamer-linked immobilised sorbent assay
AMD	Age-related macular degeneration
AMP	Adenosine monophosphate
AMPs	Antimicrobial peptides
ASPV	Apple stem pitting virus
ATP	Adenosine triphosphate
AuNPs	Gold nanoparticles
BSA	Bovine serum albumin
CE	Capillary electrophoresis
CEDIA	Cloned enzyme donor immunoassay
CE-SELEX	Capillary electrophoresis SELEX
DAB	Diaminobenzidine
DCM	Dichloromethane
DFO	1,8-diazafluoren-9-one
DMAC	Dimethylaminocinnamaldehyde
DNA	Deoxyribose nucleic acid
dsDNA	Double-stranded DNA
ECL	Electrochemiluminescence
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELONA	Enzyme-linked oligonucleotide assay
EMIT	Enzyme-multiplied immunoassay technique
FAM	5(6)-Carboxyfluorescein
FBI	Federal Bureau of Investigation
FRET	Fluorescence resonance energy transfer
FTIR	Fourier transform infrared
HCl	Hydrochloric acid
HEX	Hexachloro-6-carboxyfluorescein

HFEs	Hydrofluoroethers
HIV	Human immunodeficiency virus
HMDS	Hexamethyldisilazane
HPLC	High performance liquid chromatography
IND	1,2-Indanedione
IND-Zn	1,2-Indanedione-zinc
ISA	Individual autoantibody profile
KCl	Potassium Chloride
K_d	Dissociation constant
LIF	Laser-induced fluorescence
MALDI-MS/P	Matrix Laser Assisted Desorption/Ionisation – Mass Spectrometry/Profiling
MCAR	Mixed cell agglutination reaction
MMD	Multi-metal deposition
MW	Molecular weight
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NBT	Nitro blue tetrazolium
NCFS	National Centre for Forensic Studies
NIR	Near infrared
ORO	Oil red O
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PD	Physical developer
PEG	Poly(ethylene glycol)
PSMA	Prostate-specific membrane antigen
PVC	Polyvinyl chloride
PVDF	Polyvinylidene fluoride
QD's	Quantum dots
RCMP	Royal Canadian Mounted Police
RDT	Rapid diagnostic tests
RNA	Ribonucleic acid
RP	Ruhemann's purple
RP-Cd	Ruhemann's purple-cadmium

RP-Zn	Ruhemann's purple-zinc
RT-PCR	Reverse transcription PCR
RTX	Ruthenium tetroxide
SDS	Sodium dodecyl sulfate
SELEX	Systematic evolution of ligands by exponential enrichment
siRNA	Small interfering RNAs
SMD	Single-metal deposition
SND	Single-metal nanoparticle deposition
SPR	Surface Plasmon Resonance
ssDNA	Single-stranded deoxyribonucleic acid
TAR	Trans-activation response
TBS	Tris-buffered saline
THC	Δ^9-Tetrahydrocannabinol
THF	Tetrahydrofuran
TiO_2	Titanium dioxide
TLC	Thin layer chromatography
TMCS	Trimethylchlorosilane
TNT	Trinitrotoluene
TTBS	Tween 20 and tris-buffered saline
UK	United Kingdom
UTP	Uridine triphosphate
UV	Ultra Violet
VEGF	Vascular endothelial growth factor
VMD	Vacuum metal deposition
VSC	Video spectral comparator

Abstract

Research into latent fingermark detection and visualisation has taken many paths over the years as researchers and practitioners explore numerous methods to improve existing reagents. The majority of past research has resulted in providing small, incremental improvements to existing techniques. Currently, some researchers have opted to seek more transformational improvements in detection sensitivity, selectivity and visualisation. One such area being investigated is utilising immunology to target proteins, amino acids and drug metabolites in the latent fingermark deposit. Research to date has indicated that antibodies have great potential in providing these transformational improvements due to their ability to bind to certain fingermark components with high sensitivity and selectivity.

Following on from the antibody research, aptamers have been highlighted as the next potential immunogenic technique for several reasons, including reduced health and safety issues, lower cost, greater sensitivity and selectivity, and ease of design and versatility. Aptamers are specifically selected oligonucleotides comprised of either ribonucleic acid (RNA) or single-stranded deoxyribonucleic acid (ssDNA). Due to the selection strategies employed, aptamers can be designed to target most molecules and bind to them with detection limits in the sub-micromolar to nanomolar ranges. Although aptamers have been successfully used in a variety of highly sensitive and selective detection devices, they have not been investigated for use in the detection and visualisation of latent fingermarks prior to this project.

Initially, this project focussed on aptamers targeting amino acids as a means of visualising latent fingermarks. However, it was found that strong, non-specific interactions occurred with both the aptamer and the fluorescent tag, resulting in a lack of success with this approach.

In order to address these issues, aptamers selected to the protein lysozyme were used on fingermarks placed on both PVDF and plain white copier paper. Lysozyme was selected as it was found to be a component in human sweat, while aptamers selected to lysozyme, with binding affinities in the nanomolar range were available. It was found that the aptamer-based reagents possessed high levels of sensitivity with the clear detection of lysozyme at very low concentrations (1 ng). Latent fingermarks from various donors were able to be detected on both substrates, with primary and secondary level detail being clearly visible. Results,

however, were very inconsistent, with marks older than a couple of days being difficult to detect. This was found to be due to the degradation of lysozyme in the latent fingermark. Unfortunately, aptamers to other, possibly more suitable, fingermark components that would circumvent this problem were not available for this project. Despite the difficulties encountered, this project has, for the first time, demonstrated the potential of detecting and visualising latent fingermarks with an aptamer-based reagent. The study has laid the groundwork for future successful investigations that exploit the benefits of aptamers while overcoming the limitations identified in this project.

Chapter 1:

Introduction

Chapter 1: Introduction

1.1 Fingerprints

1.1.1 Introduction to fingerprints

Friction ridge skin is the term used to describe the complex series of ridges and valleys that produce the detailed friction ridge skin patterns as seen, for example, on the fingers. These ridges and valleys are found on humans and most primates only on the palmar and plantar surfaces of the skin. One reason for these ridges and valleys is to allow for a stronger grip and to reduce the possibility of slippage when handling items or walking [1].

Skin is composed of two main layers: the epidermis, which is the layer closest to the surface, and the dermis, the layers of the inner skin. The exact formation of the friction ridge skin detail at the surface is dependent on a layer of cells that separates the epidermis and dermis called the dermal papillae [2]. The dermal papillary ridges develop in the foetus and, between 11 to 20 weeks of estimated gestational age, the friction ridge skin across the fingers of the foetus develops. Once developed, the dermal papillary ridges do not alter throughout a lifetime, hence causing the patterns of the friction ridge skin to remain unchanged. Only through damage to the basal layer, the layer closest to the dermis, will there be permanent alterations to the skin pattern (e.g. scarring). Superficial damage will not generate permanent changes within the papillary ridges [3].

Also found within the skin structure are perspiration pores. These pores are ducts connected through the epidermis to a sweat gland beneath the dermis layer. The reason for these pores is to improve friction along the ridges of the skin, but more importantly to remove waste and to help cool the body. This is done by sweating, a process that involves a sweat gland perspiring a mixture of chemicals from within the body out on to the friction ridges. This causes a thin layer of sweat to cover the ridges of the friction ridge skin, which when coming into contact with a surface would deposit the film as an image of the friction ridge skin. However as ridges are moist they can easily pick up other contaminants from around the body as well as extraneous material. Therefore when a friction ridge skin image is deposited it

is usually a complex mixture of perspiration, contaminants and other material (see Section 1.1.5).

The patterns of the friction ridges on each finger can be classified based on the overall flow of the ridges these being loops, whorls or arches (Figure 1-1). Loops are recognised by the ridges of the centre forming a tight bend towards the distal end of the finger. Every loop has a delta (where the ridges diverge) and a core (centre of the pattern) and are classified as either ulnar loops, where the loop opens towards the little finger, or radial loops where the loop opens towards the thumb. Whorls form central spiral patterns, which range from being tight circular patterns to extended cores and always contain at least two deltas. Of the four basic pattern groups, arches have the simplest form with the ridges running across the finger from one side to the other rising in the middle and without making a backwards turn [4-7].

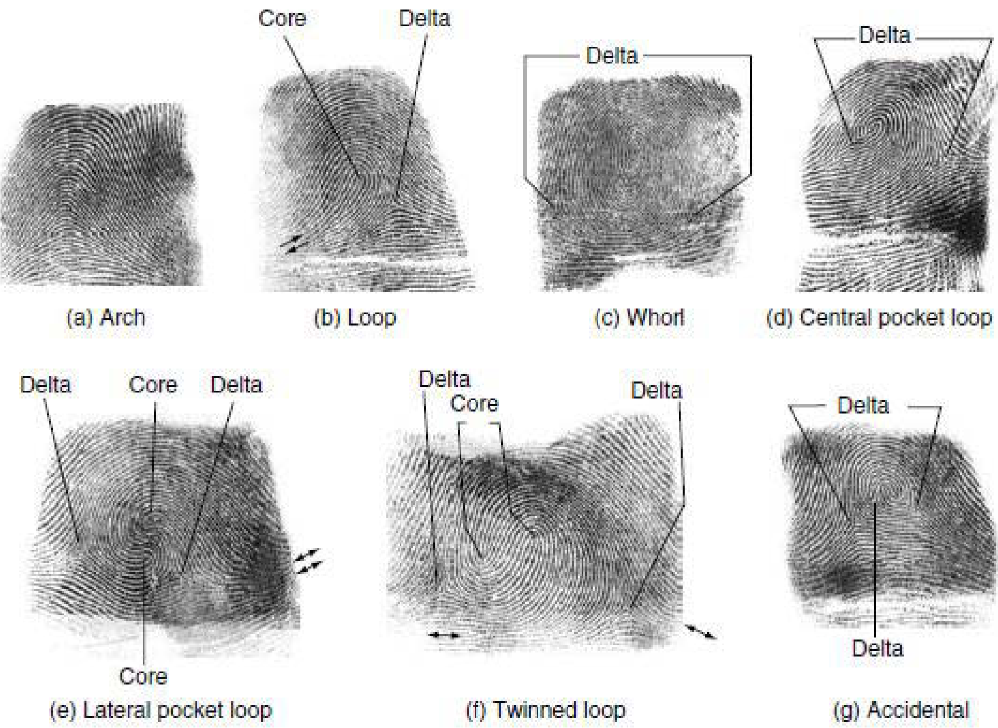


Figure 1-1: Examples of an arch (a), loop (b) and whorl (c,d,e,f,g) fingerprint. Taken and reproduced from Clegg [7].

Further discrimination of the ridges can be made by the examination of the ridge characteristics or minutiae. The ridges of friction skin do not run continuously, they either end or bifurcate. These minutiae can create further characteristics as listed below and illustrated in Figure 1-2 [7]:

- Ridge endings
- Bifurcations, either singular, double or tri
- Short ridge
- Enclosure, or lake
- Spur
- Dot

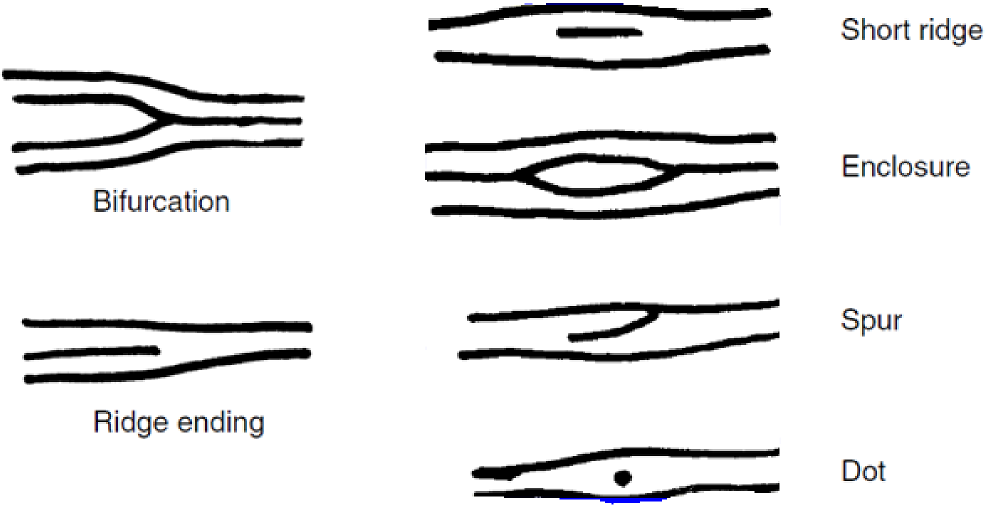


Figure 1-2: Illustration of the various minutiae possible in a fingerprint. Taken and reproduced from Clegg [7].

Although some of the same characteristics may be present on a number of different fingerprints, the organisation and arrangement are highly polymorphic and often considered unique to the individual finger. Fingerprints have been used as a form of identification evidence for over 110 years. The first use of fingerprints as evidence came in 1892 in a murder in Argentina where the murderer was identified by comparing her fingerprints against those found at the crime scene [8]. Since the early 1900s, records of fingerprints have been stored in databases. These databases have grown massively through the years with the FBI's own collection being at almost 50 million fingerprint records. For more discussion on the history of fingerprints see Section 1.1.2.

Before progressing further, it is necessary to define the terminology relating to friction ridge skin that is used throughout this thesis. There needs to be a clear distinction between the terms fingerprint and fingermark. This has been described in a discussion of fingerprint terminology by Champod *et al*. [3]. “Fingerprint” is used only to describe a comparison or recorded print of an individual’s friction ridge skin pattern taken under controlled conditions for identification, exclusion or database purposes. “Fingermark” on the other hand refers to the inadvertent or uncontrolled placement of a friction ridge pattern on a surface. Fingermarks include those that are visible, plastic and latent together with those marks that are partial, distorted and reversed [3].

1.1.2 History of fingerprints

The oldest known uses of friction ridge skin date back to 3000 BC from carvings found on artworks in various tombs that illustrate an understanding of the unique patterns of friction ridge skin [9]. The use of friction ridge skin as a means for identification and individualisation is first known to have occurred in China around 221 BC. Fingerprints were first used on clay seals as a means to show authorship and prevent tampering, while with the invention of paper, fingerprints were commonly used to sign various legal documents [9]. In the western world, however, it was not until the late 17th century that European scientists began to take an interest in friction ridge skin with three separate scientists publishing research within three years of each other.

In 1684, Dr. Nehemiah Grew wrote about the ridges and pores of the hands and feet, while in 1685 G. Bildoo described sweat pores and textured ridges [9]. With Marcello Malpighi, who some regard as the first histologist, writing about the human fingertips varying ridges and patterns of loops and whorls in 1686 [10]. Although considerable research had been undertaken with friction ridge skin it was not until 1788 that its uniqueness characteristic was realised by J.C.A Mayer, who is credited as being the first person to state that friction ridge skin is unique [9]. The first classification system of friction ridge skin patterns was undertaken in 1823 by Johannes Evangelista Purkinje who described nine categories of patterns and gave rules for their interpretation [11]. This work laid the foundation for the successful Henry system as described later.

From this early work, research continued strongly, mainly through the separate work of two men, Sir William Herschel and Dr Henry Faulds. Both men have been classed as the pioneers

of friction ridge skin identification due to their detailed work. Herschel is credited as being the first person to officially use fingerprints on a large scale. While working for the civil service of India, Herschel used handprints as a means of personal identification on legal documents and contracts, thus developing a technique for stopping fraud and false impersonations with respect to government pensions. He is also credited with being the first person to show ridge persistency through the examination of his own prints taken at various times of his life [5]. At the same time as Herschel, a Scottish medical missionary, Dr Henry Faulds, identified that the oil and sweat from the pores in the fingers produced latent marks that could then be subsequently developed through the use of various powders. With this, Faulds was able to prove a man innocent in a burglary case as his prints did not match the marks found on the inside of a broken window. This credited Faulds with the first publication of the value of friction ridge skin for individualisation, especially in its use as evidence [9].

In order for fingerprints to become a reliable method of individualisation, further work in classifying prints was needed. This was achieved through work by Sir Francis Galton in England, Sir Edward Henry in India and Juan Vucetich in Argentina. Galton published a book entitled *Finger Prints* in which he set forth practical methods for fingerprint recording and classification. In some parts of the world today, various areas of fingermarks are still referred to as Galton features [12]. Having read Galton's work, Vucetich designed and implemented his own identification system in 1891. The success of this system can still be seen today as it was still implemented in South America [11] and in some European countries until recently. Henry in the meantime furthered Herschel's work and developed a system of fingerprint classification and filing. This he called the Henry system, which was implemented in England and Wales in 1901, and with certain modification was still in use until the end of the 20th century [10].

From these early beginnings, friction ridge skin patterns have since become a pillar in the identification of individuals. They have been used for over 110 years and are one of the most widely used and dependable methods for individualisation used by law enforcement agencies [13]. Although in recent times the use of certain individualisation methods (including fingermarks) has come under scrutiny in reports such as the National Academy of Sciences [14], extensive research and numerous court proceedings have proved the admissibility of fingermark evidence [15-21]. This acceptance of fingermark evidence is based on three established premises as identified by Moenssens *et al.* [22]:

1. Friction ridge patterns that develop during foetal life will remain unchanged throughout life and even after death until decomposition destroys the skin;

2. Friction ridge skin patterns differ from individual to individual and also from digit to digit, and are never duplicated in their minute detail; and,

3. Even though friction ridge skin characteristics are distinct, the overall appearance of friction ridge skin patterns have similarities that allow for a systematic classification of the impressions.

This classification of the overall patterns of friction ridge skin forms the basis of identification. However, individualisation cannot be achieved through just the general pattern; it requires a full and in-depth study of the fingerprint's distinct ridge characteristics referred to as minutiae.

1.1.3 Friction ridge identification

As has already been stated, fingerprint evidence is admissible in courts of law around the world as a form of identification evidence due to the uniqueness that is found in friction ridge skin. In nature, patterns are never repeated in their morphological structures, which can be explained in part through biology, chemistry and physics and by the thorough study of natural patterns [23]. This premise can be applied to the uniqueness of friction ridge skin and is elegantly summarised by Ashbaugh:

> "[T]he friction ridges are constructed of ridge units. The number of ridge units that make up a ridge is established at random. Where one ridge starts and stops, the factors that designate its length are completely dependent on differential growth. The location of the ridge unit where a branching develops is also established at random. Due to the plethora of genetic and physical variances the ridge units are subjected to during ridge formation and the number of units involved, the paths of friction ridges are unique to that area of friction skin" [24].

In determining identification, Dr. Edmond Locard, in 1914, presented his conclusions and the criteria that should be followed to provide reliable identification based on statistical analysis carried out by Balthasard and Galton. His work lead to the tri-partite rule as summarised below:

1. If more than 12 concurring points are present in the fingermark and it is of a sharp quality, then identity is certain beyond debate;

2. If 8 to 12 concurring points are present, then identity is borderline. Certainty is dependent on a number of points;
    a. The sharpness of the fingermarks;
    b. The rarity of the type;
    c. The presence of the core and delta in the exploitable part of the print;
    d. The presence of pores;
    e. The perfect and obvious identity regarding the width of the papillary ridges and valleys, the direction of the ridges and the angularity of bifurcations; and,

3. If there are only a small number of characteristic points available in the mark then certainty of identification cannot be made. Only a presumption is possible proportional to the number of points available and their clarity [25].

Locard's tri-partite rule is still used in parts on the world today and is the basis for the ACE-V method used nowadays in determining an identification. Currently, fingerprint examiners usually follow a four-step process called ACE-V (analysis, comparison, evaluation and verification). This methodology was first developed and adopted for fingerprint examination by the Royal Canadian Mounted Police (RCMP) and is briefly discussed below. The interested reader is directed to the following references for a full explanation [3, 5, 23].

In the initial analysis stage, a full in-depth assessment of the ridge formations and the clarity of the latent mark is undertaken. During the analysis stage, only the latent mark is investigated allowing for total objectivity. Information that is visible and reliable is gained from the mark, taking into account the clarity of the image but also effects such as distortion, pressure, surface type and development technique [3]. At this stage, information from the friction ridges can be divided into one of three levels (these are described below as they will

be referred to throughout this thesis). Once the recovered mark has been thoroughly annotated by highlighting all the feature levels, it can then be compared with the known print. This will highlight all the concordances between the two marks but also any differences. Evaluation of the comparison is then undertaken where the examiner must logically evaluate the two marks, assessing both similarities and differences. One unexplainable dissimilarity can be enough for an exclusion irrespective of the number of concordances [3]. If there is agreement between the two marks and with sufficient detail in agreement to eliminate all other possible donors, then an identification can be made [6]. Verification is the last stage and is undertaken by a second fingerprint examiner undertaking all the previous steps.

The levels of detail used in the analysis of fingermarks was first introduced by Ashbaugh to describe the various types of information available in a fingermark [5]. The visualisation of these different levels of detail relates to the clarity of the mark; as the levels increase, the clarity of the mark must also improve [23].

First level detail – This refers to the overall pattern of the mark, the general direction of the ridge flow, but is not limited to the defined classification of fingermark patterns (see Section 1.1.1).

Second level detail – This involves the actual path of a specific ridge – where the ridge starts, where it stops, the length and what it does. Ridge characteristics or minutiae (see Section 1.1.1) are part of secondary level detail. The ridge paths with length and terminations are highly variable. This level of detail can only exist with first level detail present.

Third level detail – This level of detail is involved with the shapes of the ridge structures including the morphology (pore positions, edges and textures) of the ridges. These features are also highly variable but are the most difficult to observe due to the minute scale of the detail.

With these terms relating to the clarity of a fingermark, they in turn can be used to describe the successfulness of a latent fingermark detection technique. It must also be noted that the success of any detection technique is also impacted by the way the fingermark is deposited. Throughout this thesis these levels of detail will therefore be used to describe the effectiveness of the fingermark reagents tested.

1.1.4 Types of fingermarks

Ridgemarks are created when a person makes contact with a surface by a fingermark or palmmark. The way this mark is made, however, can differ by the type of surface on which the mark is placed upon, or, through the material transferred to form the mark. There are three main ways in which latent fingermarks are deposited as described below:

Visible – are fingermarks that are easily visible to the naked eye. This is normally due to ridge detail being transferred by a foreign substance, such as blood or grease, onto the surface.

Plastic – fingermarks that result from force being applied from the finger onto a soft surface such as putty or wax. Plastic fingermarks will be reversed, with the ridges of a finger being the valleys in the resulting three-dimensional impression.

Latent – are fingermarks that are invisible to the eye, but that happen to be the most commonly recovered fingermark type at a crime scene or on an item of evidence. Latent fingermarks are transferred to the surface through bodily secretions and contaminants found on the fingers (see Section 1.1.5). Through these transferred materials, latent fingermarks can be visualised via optical, physical or chemical development to allow for examination and comparison with fingerprint records [2] (see Section 1.1.7).

1.1.5 Latent fingermark composition

Latent fingermarks are the result of natural perspiration from the pores of the skin and external contaminants picked up through the touching of foreign objects. There are five possible sources that participate in latent fingermark composition. These are:

- eccrine perspiration;
- apocrine sweat;
- sebaceous secretions;
- epidermic substances; and,
- external substances [26].

The human body has three types of sweat glands located in different areas responsible for secretion. Each gland is responsible for the secretion of various inorganic and organic materials. These three glands are the:

- sudoriferous eccrine;
- sudoriferous apocrine; and,
- sebaceous glands [27].

When a finger touches a surface, only a few micrograms of the material found on the fingers is transferred to the waiting surface [28]. Latent fingermark composition is extremely hard to study over a general population due to the fact that each individual does not excrete the same components. This is compounded by the fact that the amounts at which each individual excretes these components can vary hugely dependent on a number of factors including temperature and health. Fingermark composition is further complicated by a range of foreign contaminants that may be found in a mark. These contaminants can range significantly as they are picked up as a person touches any surface before making the mark.

The relative abundance of amino acids (secreted by the eccrine glands) in fingermark deposits was summarised by Knowles [29]. Through four studies, the most abundant amino acid was found to be serine with glycine second and ornithine third (Table 1-1). The concentration of amino acids in sweat has been found to be between 0.3-2.59 mg/L. However, the amount and concentration of the amino acids depends on the individual and their health, diet, gender and age [30]. A different study into the water soluble constituents in sweat identified and quantified the following substances: calcium (0.03 to 0.3 µg), urea (0.4 to 1.8 µg), lactic acid (9 to 10 µg), amino acids (1 µg), chloride (1 to 15 µg), ammonia (0.0 to 0.3 µg), phenol (0.06 to 0.25 µg), sodium (0.2 to 6.9 µg) and potassium (0.2 to 5 µg) [31].

The composition of sebum excreted by the sebaceous glands has also been studied. Squalene and cholesterol have been identified in all fingermarks, with several squalene derivatives, fatty acids and wax esters also being identified [26]. In a separate study, squalene was found to be the most abundant followed by oleic acid, palmitoleic acid and palmitic acid [32]. Croxton *et al.* found that the main fatty acids included octanoic acid, decanoic acid, dodecanoic acid, tetradecanoic acid, pentadecanoic acid, hexadecanoic acid, octadecanoic acid, octadecanoic acid, *cis*-9, *cis*-12-octadecadienoic acid, nonadecanoic acid and tetracosanic acid [33].

Table 1-1: Summary of four studies into the relative abundances (serine ratio) of amino acids in fingermark deposits, adapted from Knowles [29].

Amino acid	Hamilton [34]	Hardorn *et al.* [35]	Oro and Skewes [36]	Morgan [37]
Serine	100	100	100	100
Glycine	67	54	59	54
Ornithine	32	45	45	40
Alanine	27	35	28	22
Aspartic acid	22	11	22	24
Threonine	17	9	18	18
Histidine	17	13	14	16
Valine	12	10	9	7
Leucine	10	7	10	7
Isoleucine	8	6	8	6
Glutamic acid	8	12	5	3
Lysine	10	5	-	6
Phenylalanine	7	5	5	5
Tyrosine	6	3	5	4

Although proteins are known to be present in human sweat, only a few have been identified in latent fingermarks (Table 1-2). Drapel *et al.* highlighted proteins in fingermarks due to desquamation in the skin's regeneration process [38]. These proteins were keratins 1 and 10, cathepsin-D and dermcidin. In a recent study using matrix assisted laser desorption ionisation mass spectrometry profiling (MALDI-MSP), a further protein psoriasin has been identified as being a component of latent fingermark residue, however, it was not able to be quantified [39].

Table 1-2: Overall amount of protein detected in a swab of both hands and in one fingermark, adapted from Drapel *et al.* [38]. Note the data for one fingermark has been extrapolated considering a surface area of 1 to 82 between both hands and one finger.

	Both hands	(mg)	One finger	(µg)
	Mean	Min/Max	Mean	Range
Eccrine	10.9	10/16	133	128-190
Charged	31.4	20/44	384	243-542

1.1.6 Deposition factors and substrate characteristics

There are a number of deposition factors that can affect how a fingermark is first placed and then remains on a surface. These include the conditions surrounding the contact between the friction ridge skin and the surface and are well summarised by Yamashita and French [40] and Olsen [41] as highlighted below:

Pre-transfer conditions – This refers to the health of the fingermark donor's ridge skin and the amount of residue present. These conditions are highly variable due to age, sex, occupation, disease and the contamination of fingermark residues through a range of substances that can be picked up by the fingers.

Transfer conditions – This includes the condition of the substrate on which a fingermark is placed such as the texture, shape, curvature, surface contaminants and surface temperature. The pressure applied during the deposition of the latent mark is also a transfer condition.

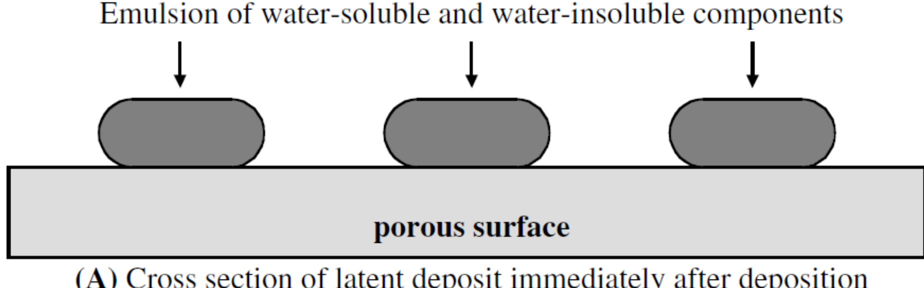
Post-transfer conditions – Often referred to as environmental factors, these play a huge role in how well a mark can be detected after being placed. These include conditions such as temperature, humidity, water, exposure to sunlight/UV and contact from another source.

As has already been seen above, the substrate on which marks are placed plays a key role in the ability to detect and enhance latent fingermarks. Therefore it is necessary to understand the characteristics of the substrate on which the latent fingermark is placed in order to obtain optimal development. Although this is a crude characterisation, substrates are usually grouped into three main types, porous, semiporous and nonporous. A porous surface has the ability to absorb the latent fingermark very quickly into the surface such as paper and cardboard. Nonporous surfaces do not absorb any of the fingermark compounds, the

compounds remain on the surface, examples being plastics and glass. Semiporous surfaces are unique as they have characteristics of both porous and nonporous surfaces, being slow to absorb the water-soluble organic compounds while leaving the sebaceous fat-soluble compounds on the surface. Some semiporous surfaces include waxed wrapping paper, coated cardboard packaging, stained wood, latex gloves, expanded polystyrene, treated leather and polymer banknotes.

When a latent fingermark is placed on a porous surface, the water-soluble compounds are absorbed into the first few layers of the substrate in seconds. During the absorption the water component of the eccrine sweat evaporates leaving behind a mixture of organic and inorganic compounds including amino acids, urea, salt and chlorides (Figure 1-3). These types of latent fingermarks once absorbed are relatively stable and well preserved; they cannot be damaged by contact with other surfaces. When marks contain only eccrine secreted material, however, they can be obliterated when coming into contact with water [3].

When a finger makes contact with a nonporous surface, an emulsion of water and fat-soluble constituents is formed on the surface and may remain there for a considerable amount of time (Figure 1-4). They are, however, delicate due to all of the constituents remaining on the surface; they can be easily damaged or rubbed off. Semiporous surfaces absorb the water-soluble compounds into the surface but at a slower rate than porous surfaces. The fat-soluble compounds will remain on the surface but for less time than with nonporous surfaces.



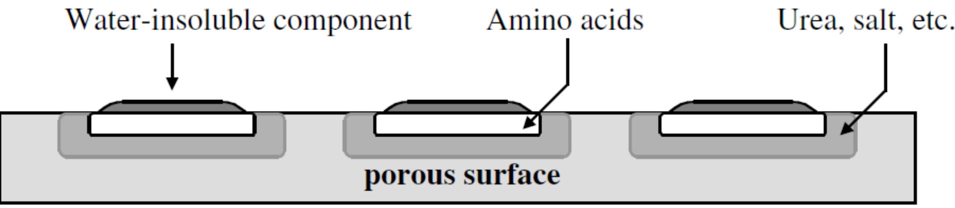
(A) Cross section of latent deposit immediately after deposition

Water-insoluble component

Water-soluble component

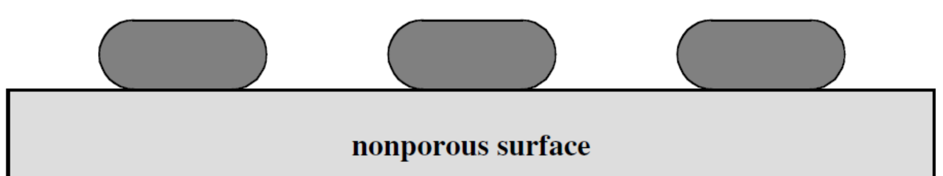
porous surface

(B) Minutes/hours after deposition

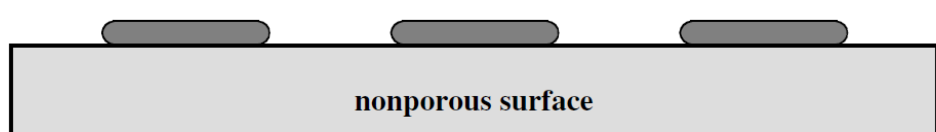


(C) Weeks/months after deposition

Figure 1-3: The effect of time on the deposition of latent fingermark components on porous surfaces. Taken and reproduced from Champod *et al.* [3].



(A) Cross section of latent deposit immediately after deposition



(B) Several weeks/months after deposition

Figure 1-4: The effect of time on the deposition of latent fingermark components on nonporous surfaces. Taken and reproduced from Champod *et al.* [3].

1.1.7 Current detection and enhancement of latent fingermarks

Detection and enhancement of latent fingermarks has been researched for well over 100 years. Over this time, scientists and investigators have strived to develop new techniques or make improvements to existing methods in order to improve sensitivity and thus detect a higher number of latent fingermarks on any surfaces on which marks may be found. Due to this research, numerous techniques have been put forward for the detection and visualisation of latent marks, both at a crime scene and in the laboratory. Some techniques have proven to be unsuitable for casework due to deficiencies including low sensitivity and high toxicity. Other techniques, however, have led to great improvements in the field of detection and visualisation.

To visualise latent marks, any technique, whether it be physical, chemical or visual, is usually targeted at some component within the latent print residue. This is to basically create a contrast between the mark ridges and the substrate and any method which meets this criterion is worth considering. Generally, current techniques may target the water-soluble components with many targeting amino acids, while other techniques focus on the sebaceous components such as the glycerides, fatty acids and squalene. Choosing the correct sequence of complementary techniques to provide the best chance of detecting any latent fingermarks present then depends on a number of circumstances including the surface to be examined, possible contaminants in the mark and on the surface, the age of the mark and environmental conditions.

Optical examination is the starting point for any possible detection and visualisation of latent fingermarks both at a scene of crime and in the forensic laboratory. Using light in the form of diffused reflection or ultraviolet can highlight latent marks before any specific treatment [3]. The use of high intensity light sources and ultraviolet light is routine after a chemical or physical detection technique has been applied to an exhibit in order to enhance the visibility of the mark. As discussed later (see Section 1.1.7.1), many techniques utilise the properties of fluorescence to achieve greater sensitivity and overcome problems such as contrast with interfering background.

1.1.7.1 Detection of latent fingermarks on nonporous surfaces

Detection and visualisation of latent marks on nonporous surfaces can be done in a number of ways. The use of powders is generally the simplest and most commonly implemented method at a scene of crime. The technique is a physical method where the powder is attracted to the moist, oily components of a latent mark deposit. Marks become visible due to the colour of the fingerprint powder creating contrast between the background surface and the fingermark residues. There are various types of powders available depending on the circumstances in which the exhibit may be found. These include granular, flake, fluorescent and magnetic powders [42].

Small particle reagent can be referred to as a liquid powder method. It again relies on the adherence of fine particles – held in an aqueous suspension – to the sebaceous deposits. It is particularly useful on fingermarks found at a crime scene where the surface has become wet or damp and where dry powder is not suitable. In the same context, powder suspensions are increasingly being used on some difficult surfaces including the adhesive side of tape, textured and plastic surfaces, as well as certain foods [43-46]. As a complement to standard dry powder techniques, various aerosol sprays containing the powder are now widely available allowing a more consistent and controlled application.

The most used laboratory based development technique for nonporous surfaces is cyanoacrylate fuming. When heated, the cyanoacrylate turns into a vapour that reacts with certain sebaceous and eccrine deposits, forming a white polymer along the ridges of the mark. The polymer formed by this polymerisation of cyanoacrylate is called polycyanoacrylate [3]. This polymer is relatively stable and allows for further enhancement by the application of a luminescent stain. There are a number of different stains available including basic yellow 40, rhodamine 6G and near-infrared dyes (see Section 1.1.8.1), all of which help to improve visualisation on difficult surfaces.

A number of stains are available for the enhancement of bloody marks on both nonporous and porous surfaces including amido black (acid black), luminol and leucocrystalviolet [47]. The most widely used techniques are amido black and diaminobenzidine (DAB). Both techniques require the blood marks to be fixed with either methanol or 5-sulfosalicylic acid prior to treatment to ensure that the blood is not destroyed by the reagents [3]. Amido black is absorbed by the proteins within the blood to produce dark blue ridges on a light blue

background [3]. Diaminobenzidine reacts with hydrogen peroxide when catalysed by the peroxidase activity of haemoglobin to produce bloody marks with ridges of a dark brown colour on a lightly stained background [3].

Together with the techniques mentioned previously, there are a number of other techniques that have proved useful under certain circumstances. Multimetal deposition (MMD) is a highly sensitive technique allowing development of marks on both porous and non-porous surfaces. Vacuum metal deposition (VMD) is a second metal deposition technique that allows the detection of marks on surfaces including plastics, glass and certain fabrics [48]. It can be very useful on old marks and those that have been exposed to harsh environmental conditions [3].

1.1.7.2 Detection of latent fingermarks on porous surfaces

In detecting latent fingermarks on porous surfaces, the vast majority of detection is done within a forensic laboratory. This is due to the nature of porous surfaces. The porous surfaces absorb the fingerprint residues in a very short time so techniques such as powders will only be useful on marks that are fresh. There are more suitable and more sensitive techniques that can be applied on porous items once in a laboratory.

Ninhydrin is one of the oldest but most widely used methods for latent fingermark detection and visualisation on porous surfaces. It involves a reaction with the primary amines including amino acids, proteins and peptides. These compounds are one of the main organic constituents of perspiration. The amino acids are absorbed by the porous surfaces and remain relatively stable thus allowing a reaction with ninhydrin to take place. The result of the reaction with these compounds is a visible fingerprint with non-fluorescent purple ridges. This purple compound is referred to as Ruhemann's purple (RP) [49, 50].

The generally accepted reaction mechanism for ninhydrin is that reported by Grigg and co-workers [51]. When reacted with an amino acid, ninhydrin tautomerizes to 1,2,3-indanetrione forming a Schiff's base. This resultant Schiff's base then undergoes decarboxylation and cleavage to form a resonance-stabilized 1,3-dipolar species. Through proton transfer, an intermediate aldimine is formed that is hydrolysed to an aldehyde and a 2-amino intermediate. The Ruhemann's purple product is produced by condensation of the intermediate with a second molecule of ninhydrin [52] (Figure 1-5).

Figure 1-5: The reaction mechanism for the production of Ruhemann's purple. Taken and reproduced from Grigg *et al.* [52].

Ninhydrin is a very successful technique; however, it can suffer from contrast and sensitivity issues. Standard ninhydrin treatment gives non-fluorescent marks and produces only the purple product, therefore limiting its uses on dark backgrounds. It is also a poor reagent on porous surfaces that have specific coatings [53]. Contrast issues can be resolved by the addition of a secondary treatment using metal salts, either zinc or cadmium, which forms a complex with Ruhemann's purple. RP-Zn forms an orange coloured mark while RP-Cd gives a red product. Both of these give Ruhemann's purple luminescence properties when viewed at liquid nitrogen temperature (-196 ^{o}C) [54]. These properties can be very useful when viewing marks where surface contrast in the absorption mode is a problem [3].

Together with the addition of metal salts to improve ninhydrin characteristics, there have been numerous research papers aimed at changing the chemical structure of the reagent to introduce fluorescence and other properties. There have been many structures proposed and examined, nearly 100, of which some have shown great promise. 5-Methoxyninhydrin exhibits similar results to ninhydrin under normal light however, after zinc chloride treatment, it has strong room-temperature fluorescence [53]. Benzo[f]ninhydrin was found to produce much stronger fluorescence after zinc salt treatment together with a quicker reaction time under ambient conditions. However, benzo[f]ninhydrin produced less contrast than ninhydrin while the fluorescence produced is similar to that of 1,8-diazafluorene-9-one (DFO) and other ninhydrin analogues [55]. Thieno[f]ninhydrin has also shown a good

combination of chromogenic and fluorogenic properties, again after zinc chloride treatment [53].

DFO is an amino acid targeting technique developed from research into ninhydrin. It has been shown to react in a similar manner as ninhydrin (Figure 1-6), developing a visible latent fingermark [56, 57]. When in contact with an amino acid, DFO forms an imine; under decarboxylation and hydrolysis this forms an aromatic amine. Reaction of this amine with excess DFO results in the formation of a red product [58]. DFO produces ridges that are pale red to purple in colour but have an intense luminescent character at room temperature under an alternative light source or laser.

Figure 1-6: Proposed reaction mechanism of 1,8-diazafluorene-9-one with L-alanine. Taken and reproduced from Wilkinson [56].

A third amino acid targeting technique is 1,2-indanedione (IND), which was first publicised as a latent fingermark detection technique in 1997 [59]. IND reacts with amino acids to give a similar result to DFO, forming ridges of a pink colour, while producing bright luminescence at room temperature under an alternative light source or laser. The reaction of IND with amino acids again behaves in a similar manner to ninhydrin, with the initial reaction with amines creating imines; after decarboxylation and Strecker degradation, 2-amino-1-indanone is produced (Figure 1-7). Further reaction with excess IND produces the pink coloured ridges

and strong luminescence properties [58]. IND has been found to be very sensitive to environmental conditions with optimisation needed for different jurisdictions. However, it displays the greatest sensitivity and limits of detection of the three main amino acid targeting reagents [60].

Figure 1-7: Reaction mechanism of 1,2-indanedione with α-amino acids. Taken and reproduced from Jelly *et al.* [58].

Since the emergence of IND for latent fingermark detection in 1997, the technique has been the subject of constant research to both understand its mechanisms and optimise its performance. It has been found to cause varying results depending on the geographical location in which it has been used [61, 62]. This is due to differences in formulations of IND, effects of relative humidity and possible effects of porous substrate constituents on the reaction between amino acids and IND. An optimum IND reagent has recently been developed with the addition of zinc to the solution [63]. The indanedione-zinc (IND-Zn) formulation was found to be less affected by humidity with similar results being developed in relatively low humidity to those developed in humidity above 70% [63]. Further research into IND formulations has been completed by Wallace-Kunkel *et al.* [64], Wiesner *et al.* [65],

Merrick *et al.* [62] and Gardner and Hewlett [66]. The exact reaction mechanisms for 1,2-indanedione and IND-Zn with amino acids adsorbed to cellulose has also been determined by Spindler *et al.* [60, 67].

Detection of latent fingermarks on porous surfaces that does not involve targeting amino acids can be achieved by physical developer (PD). PD was developed in the 1970s and is based on photographic chemistry [68]. The technique targets the non-water soluble components in a latent fingermark residue. This is extremely useful as it allows the development of fingermarks on porous surfaces that have been wet, which neither ninhydrin, DFO or IND/IND-Zn can achieve. PD is an aqueous solution containing silver ions, citric acid, a ferrous/ferric redox system, and a cationic surfactant, which allows for the reduction of silver onto the latent fingermark deposits creating dark grey to black ridges [3].

Oil red O (ORO) is another technique that allows for the development of latent marks on porous surfaces. The technique stains the lipid content of the mark, producing red ridges on a pale pink background [69]. The technique was found to produce better results than PD on fresh charged marks [70]. However, after approximately one month, PD was found to be more effective [71]. Nile red is another lipid stain that is luminescent under a high-intensity light source. Nile red is simpler to use than PD and has been found to work well in collaboration with PD, developing fingermarks not detected by PD and vice-versa [72].

1.1.7.3 Detection of latent fingermarks on semi-porous surfaces

Semi-porous surfaces can often be problematic for fingerprint reagents as they fall somewhere in between porous and nonporous surfaces and, as such, behave differently with porous or nonporous reagents, often producing inferior results. Semi-porous surfaces include waxed paper, glossy paper, matte-painted substrates and some rubber/latex gloves. Some techniques that have been shown to be successful to some extent include an iodine/benzoflavone spray and MMD [3]. Apart from these two options, the best approach is to apply several reagents in a logical sequence, ensuring that the reagents are tested on similar surfaces before application to the evidential item [3].

1.1.7.4 *Miscellaneous detection techniques*

Although the above mentioned reagents are the most common for use in forensic casework, there is a plethora of miscellaneous reagents available that can be of use in specific circumstances or are simply favoured by certain forensic laboratories. These reagents can be of use when dealing with fingermarks on adhesive tape or on human skin, for example, and are detailed by Champod *et al.* [3] and Ramotowski [73]. Some of the more common miscellaneous detection techniques are listed below:

- Dimethylaminocinnamaldehyde (DMAC)
- Ruthenium tetroxide (RTX)
- Silver nitrate
- Iodine/benzoflavone
- Gentian violet
- Sticky-side powder

1.1.8 The need for new fingermark development and visualisation techniques

Even though there are a considerable number of reagents and techniques available for latent fingermark detection and visualisation for use under a variety of conditions, there is still a constant search for new and improved reagents. The reasons for this have been highlighted in several discussions [74-76] and are due to most current techniques suffering from one or more of the following disadvantages:

- Insufficient sensitivity;
- Lack of detection on problematic surfaces;
- Labour intensive and time consuming;
- Environmental and safety considerations;
- Cost;
- Lack of portability; and,
- Inability to undertake further forensic examinations (eg. DNA analysis).

Furthermore, Almog and co-workers [77] have stated that “there is a considerable number of potentially case-solving latent prints that cannot be visualised by current techniques” which further highlights the lack of sensitivity and selectivity of current techniques. While Champod

et al. [3] make clear where research needs to be focused in order to develop reagents that can:

- Offer increased sensitivity and signal to noise ratio;
- Be readily deployed at crime scenes;
- Be introduced in sequences of detection techniques or in sequence with other forensic investigation methods;
- Simplify the detection process by reducing the number of steps or allowing automation;
- Reduce the overall cost of fingerprint processing; and,
- Avoid the use of hazardous chemicals.

1.1.8.1 Current research trends

Much of the research effort to date has focused on existing techniques, improving their characteristics to better suit the needs of latent fingerprint technicians [44, 53, 78-82]. However, in an attempt to produce reagents that can satisfy the conditions laid out by Champod *et al*. and reduce the potential for 'missing' case-solving latent prints, several research groups have started to explore the capability of novel techniques.

The first class of novel fingermark reagents are based on naturally occurring products. To date there have been two potential reagents of this type developed, these being genipin and lawsone. Genipin is a non-toxic and chemically safe dye used in Chinese medicines, and as a fabric colourant. It reacts with amino acids, producing blue ridges with luminescent properties [58, 77]. Lawsone (2-hydroxy-1,4-naphthoquinone) belongs to the group of compounds referred to as naphthoquinones, which react well with amino acids. Lawsone is responsible for the staining properties of henna (decorative body staining dye). In a reaction with primary α-amino acids on paper surfaces lawsone was found to produce ridges of dark purple/brown colour which also give strong photoluminescence without a secondary treatment [83]. Both genipin and lawsone, however, react stoichiometrically. In order to achieve greater sensitivity, techniques that react non-stoichiometrically are an ideal candidate to provide higher sensitivity and enhancement, especially where marks remain undetected because they have limited residues remaining.

The use of nanoparticles is an area of fingermark detection receiving a large amount of new research. Nanotechnology is centred on the manufacture of functional materials, devices and

systems using matter of nanometre dimensions (1-100nm) [84]. One of the main advantages of using nanoparticles is an increased ratio in surface area to volume compared to bulk materials. Nanoparticles range from being 1,000 to 10,000 times smaller than the width of a latent fingermark ridge thus giving much greater resolution upon detection [85]. As the size of the materials decrease, different physical phenomena become apparent including the crystal phase of the material, electron transfer and doping properties, and light interactions, therefore allowing them to easily fluoresce [84]. Nanoparticles, however, do not generally show affinity for fingermark residues so need to be modified to allow the selective detection of residues without an affinity for the background [85]. Luckily, nanoparticles can be easily modified by adding chemical functionalities or molecular chains to their outer surface [85].

Synthesised nanoparticles were first used in the MMD technique with the use of gold nanoparticles (AuNPs) and silver to develop latent marks with high sensitivity [86-88]. However, marks developed by MMD are non-luminescent therefore the use of zinc oxide as a replacement to the silver has been used to produce luminescence and thus increase the contrast between the ridges to the background [89]. In a quest to make the MMD technique simpler, two techniques, single-metal deposition (SMD) and single-metal nanoparticle deposition (SND) have been investigated. In the SMD approach, the physical developer stages are removed and replaced by a gold-hydroxylamine solution, allowing the growth of AuNPs on the fingermark ridges through a redox reaction between tetrachloroauric acid and hydroxylamine [90, 91]. In a separate approach, the use of AuNPs stabilised by glucose were used in a single step method [92]. Over a wide pH range (2.5-5), AuNPs were found to adhere to latent fingermarks when left in the nanoparticle solution for 40 minutes before excess reagent was washed off the surface to reveal development.

In a modified approach to the universally used method of physical developer, Jaber and co-workers utilised the power of nanoparticles to create a reverse development method that they state 'may increase the overall yield of developed fingermarks as it bypasses the issue of the remarkable differences in sweat composition between individual persons' [93]. This is possible due to the functionalisation of AuNPs with an active head, being a polar group that has strong affinity to cellulose, attached via a long chain to an active tail, being a sulfur group that has the ability to stabilise AuNPs. This approach causes the silver precipitate of physical developer to deposit onto the nanoparticle coated cellulose substrate rather than the fingermark residues, which is what generally happens in the conventional physical developer

approach. This develops fingermarks that are uncoloured but visible due to the dark background of the deposited silver [93].

In a study by Islam and co-workers, the use of chitosan as a bioadhesive to AuNPs was found to produce latent fingermarks on glass slides [94]. By using chitosan as a pre-treatment on the latent fingermarks, the hydrophobic chitosan was found to attach to the lipid residue. Under forced agglomeration, the AuNPs attach to the positive end of the chitosan. In current research, the use of ninhydrin thiohemiketals as an attachment method for AuNPs is being researched. It is hypothesized that thiohemiketals of ninhydrin will improve the preference of AuNPs to attach to the fingermark ridges rather than the background and therefore, after silver deposition, contrast will be improved [95].

In fact, nanoparticle conjugation systems have already highlighted their use in improving attachment to and enhancement of latent marks. Spindler found that, when un-conjugated or partially conjugated to anti-L-amino acid antibodies, AuNPs enhanced the sensitivity of the reagent [60, 96]. Becue *et al*. noted that with gold colloids functionalised with cyclodextrins and a dye solution quicker and more efficient detection of latent marks was observed [97].

The use of nanoparticles is not just confined to treatment in solution. Metal nanoparticles as powders are also an attractive area of current research. Gold and silver nanoparticle powders were developed and tested by Choi *et al*. [98]. Stabilising the nanoparticles with oleylamine coated the particles in long chain alkyl molecules giving the surface of the nanoparticles a lipophilic surface to enhance attachment with the lipid residues of the mark [98]. Titanium dioxide particles (TiO_2), zinc oxide particles, quantum dots (QDs) and cadmium sulfide nanocrystals have also been examined as nanoparticle powders due to their luminescent properties. Wade found TiO_2 to give excellent results when used as a substitute for sticky-side powder on both sides of the tape [99]. While, in a further study, Choi *et al*. used TiO_2 particles to develop a new perylene dye [100] (Figure 1-8).

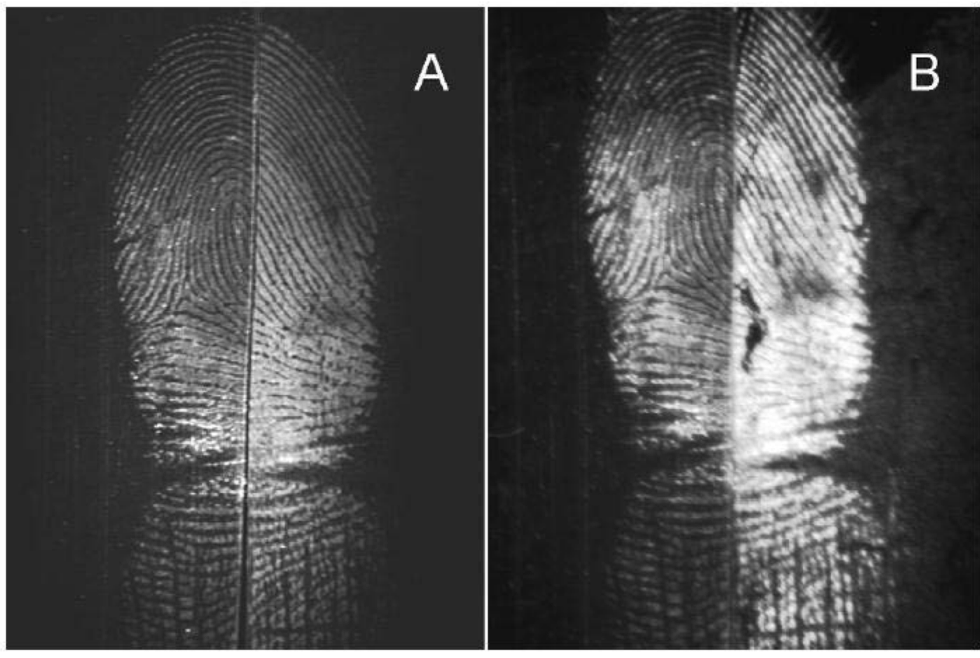


Figure 1-8: A comparison of perylene dye on TiO_2 and commercial fluorescent powders. A) perylene/TiO_2 (left), black emerald fluorescent magnetic powder (right) on polyethylene. B) fluorescent dye/TiO_2 (left), blitz green magnetic powder (right) on polyethylene. Images viewed with 575nm longpass filter and 505nm illumination. Taken and reproduced from Choi *et al.* [100].

Zinc oxide was found to develop marks with clear fluorescent ridges when illuminated at long UV wavelengths [101]. Cadmium sulfide nanocrystals have been used as a fluorescent stain on cyanoacrylate fumed marks, producing intense luminescence when viewed under the near UV lines of an Ar-laser [102]. Cadmium sulfide, when combined with selenium in a nanoparticle suspension, has been found to develop marks with strong luminescence on adhesive surfaces with a better resolving rate compared to gentian violet [103]. QDs have also been utilised in fingermark detection. QDs are semi-conductor nanocrystals which have intrinsic optical and electrical properties [104]. These include: up to 20 times brighter fluorescence than standard fluorescent dyes, emission at various wavelengths depending on the size of the nanoparticle, a broad excitation spectrum in the ultraviolet allowing for excitation by a single excitation source, tunable during synthesis and high quantum yield and resistance to photobleaching [85]. QDs encapsulated with cadmium sulfide were used as a freeze-dried powder suspension for the detection of latent marks on aluminium foil [104]. However, the starting materials needed to produce quantum dots possess a number of health and safety factors including carcinogenic properties that may be transferred to the dots themselves [105].

Silica nanoparticles represent a new possibility for improved fingermark detection due to their ease of synthesis and the silica coating of some dyes preventing photodecomposition, thus increasing photostability and emission in quantum yield of the dyes, while decreasing photobleaching [85]. Current research involving silica nanoparticles is aimed at chemically functionalising dye-doped particles to recognise fingermark residues (Figure 1-9). The addition of specific ligands to the surface of the particles allows them to selectively adhere to the fingermark residues while still providing the intense luminescence of the silica nanoparticles [85].

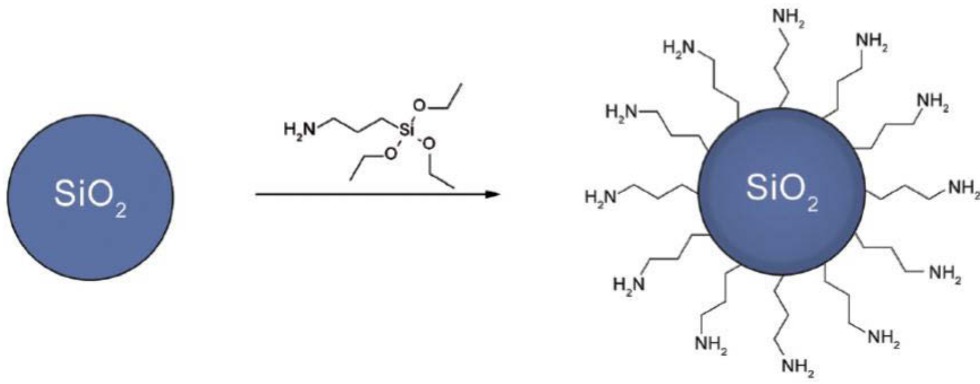


Figure 1-9: Schematic of the possible functionalisation of the outer shell of a silica nanoparticle to create interaction with organic compounds of latent fingermarks such as amino acids and proteins. Taken and reproduced from Becue *et al.* [85].

The use of up-conversion or anti-stokes luminescence is another area of research aimed at improving fingermark luminescence properties for detection. Up-converters are able to reduce background fluorescence as they can absorb long wavelength illuminating radiation and emit light at shorter wavelengths due to the rare earth complexes contained within [106]. Ma *et al*. found that when up-converters were applied as a dry powder, fingermarks could be visualised with good results on normally difficult surfaces (polymer banknotes, drink cans) [107, 108].

Near infrared (NIR) chemical imaging as a latent fingermark detection and imaging technique and near infrared dyes as replacements for conventional luminescent dyes have recently been investigated by Maynard *et al*. [109]. Chemical imaging allows for several spectroscopic techniques to be carried out, including photoluminescence emission, UV-Vis absorption, Raman scattering and infrared absorption [110]. NIR absorption and luminescence has been

shown to reveal marks developed by techniques including ninhydrin, DFO, IND, black powder, iodine and genipin. The advantages noted were that better marks were obtained on paper samples containing a watermark or printed surfaces such as magazine covers and cardboard [109]. Use of NIR dyes on both cyanoacrylate and powder developed marks provided strong luminescence without the problems of background interference that are typically encountered in the visible region.

Chadwick *et al*. combined a laser dye (Styryl 11) with rhodamine 6G as a new cyanoacrylate stain [111]. Styryl 11 was used as it provides strong luminescence emission in the NIR. The dye conjugation was able to produce well developed fingermarks when viewed in the NIR on normally difficult surfaces including multicoloured glossy cardboard and black and white barcodes [111]. The other benefit of this technique is that it is able to visualise marks both in the visible spectrum and in the NIR due to the use of both rhodamine 6G and styryl 11. The combination of the Styryl 11 and rhodamine dyes (referred to as STaR 11) was further studied as a potential fingermark development powder by Chadwick and co-workers. STaR 11 was first coated onto aluminium oxide nanopowders before being mixed with silver magnetic powder. This resulting magnetic NIR powder was found to be successful on a number of surfaces and on fingermarks of various condition with the powder outperforming Blitz Green® (a commercial luminescent magnetic powder) on textured surfaces and on older marks [112].

1.2 The immunological approach

The techniques for fingermark detection mentioned previously all focus on the use of either optical, physical or chemical techniques to detect and visualise latent fingermarks. Although both individually and in sequence these techniques detect latent marks under a wide range of circumstances, there is still the major problem as highlighted by Almog that a non-negligible number of marks still remain undetected [77, 113]. In addition, problems and issues with regards to incremental improvements, sensitivity, ease of use, reduction in steps, cost and use of hazardous chemicals still remain. In order to try and overcome these issues and look at providing more transformational improvements in relation to fingermark detection and enhancement, non-linear reagents provide a great option. The use of immunological approaches to develop non-linear reagents is a prime candidate for research in this field.

1.2.1 Antibodies

Immunoglobulins, or antibodies belong to the glycoprotein family and are a range of proteins that are able to form colloidal solutions. Although some are soluble the extent to which they can disperse is dependent on their shape, their polar properties and the solvent used for dissolution. Antibodies are biopolymers with a structure primarily made up of polypeptide chains of amino acids with a carbohydrate moiety of around 3-18% of total molecular weight [114]. Antibodies are normally found in the γ-globulin fraction of blood serum after being produced by various organs and lymphoid tissues in response to the introduction of an immunogen. An immunogen can be any substance that is capable of producing an immune response; an antigen on the other hand is any substance that has the capacity to bind specifically to constituents of an immune response [115]. Antibodies are highly specific to individual antigens, having unique structures at the antigen binding site. The section of the antigen that specifically binds to the antigen binding site is called an antigenic determinant or epitope. The size of the epitope is roughly equivalent to 5-7 amino acids. Antigens may have a single epitope (hapten), a number of epitopes of the same specificity (polysaccharides) or a number of epitopes of different specificity (proteins) [115].

Antibodies can be raised to target wide varieties of compounds. In the standard synthesis of antibodies, an animal is injected with a certain antigen to produce an antibody response within the animal. If a simple single antigen is used, monospecific or monoclonal antibodies will be produced. If a complex or mixture of antigen is used, polyspecific or polyclonal antibodies are formed [114]. Antibodies have been produced against thousands of chemical compounds including all types of biochemical compounds (lipids, carbohydrates, proteins) but also drugs, cosmetics and small synthetic peptides [115].

Antibodies are heavily used in many fields of science as a detection technique due to their high sensitivity and specificity. In clinical medicine, some uses of monoclonal antibodies include the determination of tumour associated antigens, which allow the accurate localisation of tumours in the organism, and for the direct treatment of specific tumours by carrying cytotoxic drugs [114]. Solid-phase immunoassays utilise immunology for the identification of antigens or antibodies, one commonly used example of this is in the detection of antibodies from the acquired immune deficiency syndrome (AIDS) virus with an assay called an enzyme-linked immunosorbent assay (ELISA) [115]. Other uses for antibodies in non-immunological fields are shown in the table below (Table 1-3).

Table 1-3: Other uses of antibodies in non-immunological fields. Adapted from Marchalonis [116].

Discipline	Uses
Taxonomy	Serological cross-reactions among proteins as an index of specification
Endocrinology	Monitor hormone levels in blood
Pharmacology	Detection of drug levels
Diagnosis	Identification of particular viral antigens

In order to utilise antibodies for detection and selection experiments, the possible binding between the antibody and antigen needs to be identified or confirmed visually. This can be achieved in a number of ways depending on how the selected antibody is being used in association with an antigen, but all require the labelling of the antibody, antigen or antibody against immunoglobulin with a tag or marker that can be used to qualitatively and in some cases quantitatively detect binding. The three methods of detection are with radioactive isotopes, enzymes and fluorescent probes (Table 1-4).

In detecting antibody-antigen binding, either a direct or indirect method can be used. In the direct method, the antibody against the antigen (primary antibody) is conjugated with the label before being allowed to interact with the specific antigen. The indirect method allows binding of an unlabelled primary antibody with the antigen, before a secondary labelled antibody against the primary is introduced into the reaction to bind with the antibodies and highlight binding (Figure 1-10).

Table 1-4: Commonly used antibody labels for immunochemical techniques, adapted from Thorpe and Thorpe [117].

Label	Examples	Main Uses
Fluorochromes	Fluorescein	Immunohisto/cytochemistry; flow cytofluorimetry; fluorimetric assays
	Rhodamine	Immunohisto/cytochemistry; flow cytofluorimetry
	Phycoerythrin	Flow cytofluorimetry
	Texas Red	As above
	7-amino-4-methylcoumarin 3-acetate (AMCA)	As above
	Cascade Blue *	As above
Enzymes	Alkaline phosphatase	Immunohistochemistry; Enzyme immunometric assay; immunoblotting
	B-Galactosidase	As above
	Horseradish peroxidase	As above; immunoelectron microscopy
	Glucose oxidase	Immunohistochemistry
	Urease	Enzyme immunometric assay
Radioisotope	^{125}I	Non-/competitive RIA
Electron dense	Gold	Immunoelectron microscopy
	Ferritin	As above

* Trademark of Molecular Probes Inc.

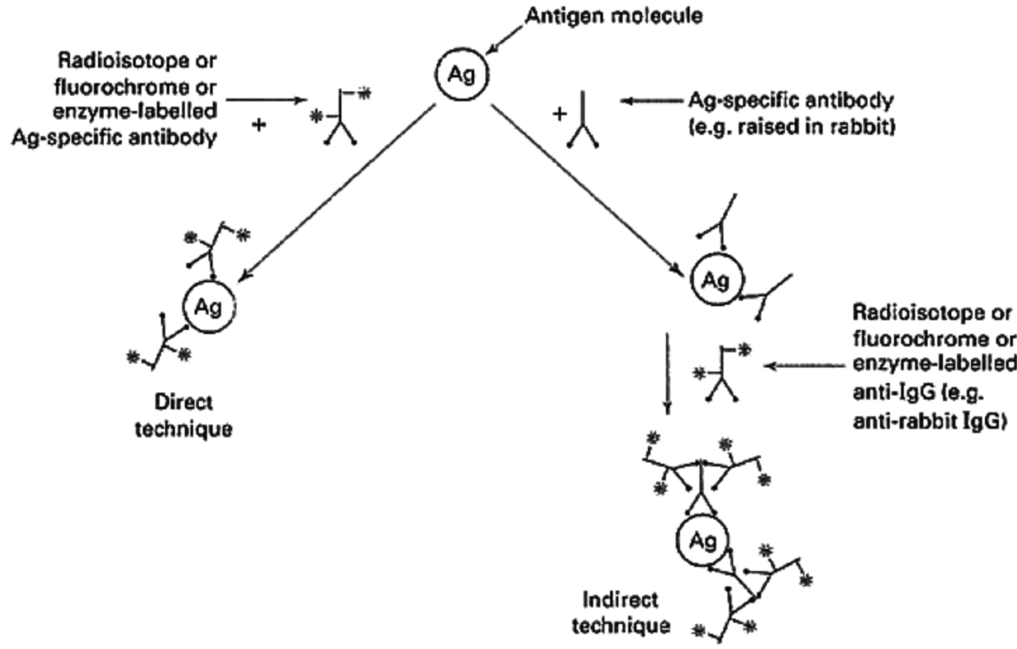


Figure 1-10: Example of antibody binding either by the direct technique (left side) or indirect technique (right side). Taken and reproduced from Thorpe and Thorpe [117].

1.2.1.1 Antibodies in forensic science

With the characteristics of antibodies, it is no surprise that they have been used in several applications relating to forensic science. In the field of forensic serology, antibodies were used for the detection and classification of questioned blood or other body fluid evidence prior to the advent of DNA profiling. The main antigenic markers in blood include the A-B-O and Rhesus +/- systems. The A-B-O system refers to the carbohydrates, present on the surface of erythrocytes, being either A, B, AB, or the O fraction (which elicits no immune response). Using either anti-A or anti-B antibodies, forensic scientists are able to determine the blood type of the sample through the agglutination of the blood to form visible red agglomerates. This is due the bivalency of the antibodies [4]. Recently the use of antibodies to detect blood at crime scenes has been proven to be of use due to the high specificity of the antibodies. By using four monoclonal anti-human antibodies, blood was able to be detected at extremely small amounts, with the detection of individual leukocytes also possible allowing for full deoxyribonucleic acid (DNA) profiles to be determined [118].

Various immunoassay techniques are also frequently used in many forensic examinations such as for the detection of drugs in various bodily fluids. Two such techniques are the enzyme-multiplied immunoassay technique (EMIT) and the cloned enzyme donor immunoassay (CEDIA), both of which are highly sensitive techniques able to identify both the drug and the amount present [4]. Roadside drug testing, currently in operation in most Australian states, is one such example of an EMIT based technique that is used in a simple, single-use drug screen kit called DrugWipe® [119] (Figure 1-11).

The use of a modified assay has been recently validated as a method for individualisation as an alternative to DNA profiling [120]. By using a specialised microarray, the technique identifies the individual specific autoantibody profile (ISA) from blood. The technique has been identified as a possible alternative to DNA-based individualisation techniques when DNA has become contaminated, while the antibody method is also able to easily individualise between identical twins, something that requires very long and expensive processes with the use of DNA.

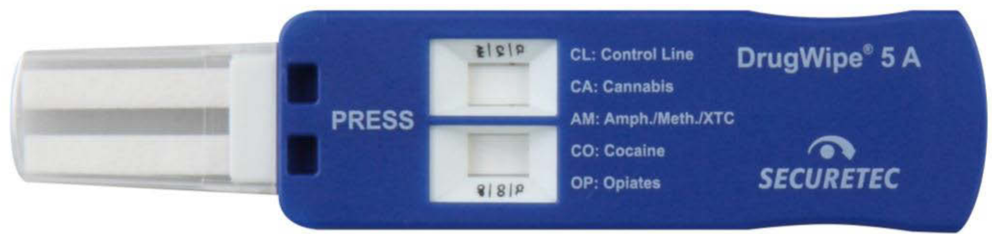


Figure 1-11: The antibody based drug screening device, DrugWipe®, able to detect the use of drugs including cannabis, amphetamines, cocaine and opiates. Taken and reproduced from Securetec [121].

1.2.1.2 Fingermark detection using antibodies

The earliest use of immunology in fingermark detection dates back to 1977 with work reported by Ishiyama and co-workers [122]. Their investigation was centred on the use of antibodies and lectins in determining isoantigenic activity (blood types) from latent fingermarks. Although detecting latent fingermarks was not the main aim of the research, they were able to demonstrate the application of antibodies for fingermark detection. Using a mixed cell agglutination reaction (MCAR), anti-A and anti-B agglutins and *Ulex* anti-H lectin were used to detect the presence of the ABH blood group material in latent fingermarks.

Initially their technique showed that it is possible to serologically demonstrate fingermark patterns impressed on the adhesive side of cellophane tape [122]. Further work on paper substrates, however, demonstrated enhanced results by using the MCAR technique as a second step after ninhydrin treatment. When used on secretor-type fingermarks on paper (20% of the given population are non-secretors), the serological staining of the latent fingermark gave a more distinct pattern than that of just the ninhydrin test as shown in Figure 1-12 [122]. This early work showed the high sensitivity of an immunogenic technique for latent fingermark detection with the authors themselves noting that the reason for the improvement in pattern contrast after using the MCAR technique was due to serological reactions being more sensitive than chemical methods such as ninhydrin [122].

Figure 1-12: MCAR technique as a second step after ninhydrin processing. Ninhydrin development alone (left) and MCAR staining after ninhydrin (right). Taken and reproduced from Ishiyama *et al.* **[122].**

Following on from this work, Pounds and Hussain further investigated the use of antibodies and lectins for fingermark detection [123]. They again focused on targeting the ABH material using commercially available anti-A and anti-B sera and various monoclonal antisera. Using the agglutination technique, they were able to detect and retrieve quality marks from both fresh and old impressions [123]. However, this initial approach had severe limitations. No marks could be detected on porous surfaces, with the most suitable substrates being polythene, polyvinyl chloride (PVC), adhesive tapes, metal foils and cellulose acetate. In initial

experiments, no marks could be detected from those donors with blood type O, although this was to be expected due to the lack of an anti-H sera. This was a major problem due to the fact that approximately 45% of the British public were group O secretors.

To overcome this problem, a range of lectins that bind to the H antigen, the carbohydrate precursor to ABO blood group antigens, were trialled together with a highly potent monoclonal anti-H sera [123]. Again using the agglutination technique, fingermarks on a range of nonporous surfaces were developed with good ridge detail. As the H antigen is present in all blood groups, including most type-O individuals, the anti-H sera produced fingermarks irrespective of blood type [123]. This approach, however, was still not without its problems. First, even with the change of target it was found that 20% of the population would still not produce detectable fingermarks as they were found to be non-secretors. Second, a common problem encountered with this method was that the red cells would bind to the entire surface on which the fingermark was placed. By adding Tween 80 surfactant to the lectin and monoclonal anti-H solutions, this was overcome [123]. The authors noted the very high sensitivity of the antibody approach demonstrated by the fact that, although minute compared to the overall amount of material contained within a fingermark, ABH as a target could still develop fingermarks with good detail. Pounds and Hussain also made reference to the fact that, if antibodies to major fingermark constituents such as triglycerides or wax esters were available, then detection sensitivity might be considerably improved [123].

Although this early work clearly demonstrated the high sensitivity and selectivity potential of an immunogenic approach for latent fingermark detection, surprisingly no further work was undertaken by either of the research groups in question. Only since 2007 have immunogenic techniques been reinvestigated as an approach for fingermark detection and enhancement. Four different research groups from around the world have reported investigations into the use of antibodies by targeting different fingermark constituents.

Drug metabolites within latent fingermarks have been successfully targeted for fingermark detection by researchers based in the U.K. Russell and co-workers have published four articles that describe the targeting of metabolites to common legal and recreational substances with the use of antibodies. In their initial work, cotinine – the metabolite of nicotine – was targeted using AuNPs functionalised with multiple anti-cotinine antibodies. This functionalisation step served to increase the specific interaction between the antibody

and the antigen [124]. Protein A was used as a biological linker to attach and orientate the anti-cotinine antibodies on the AuNP surface (Figure 1-13).

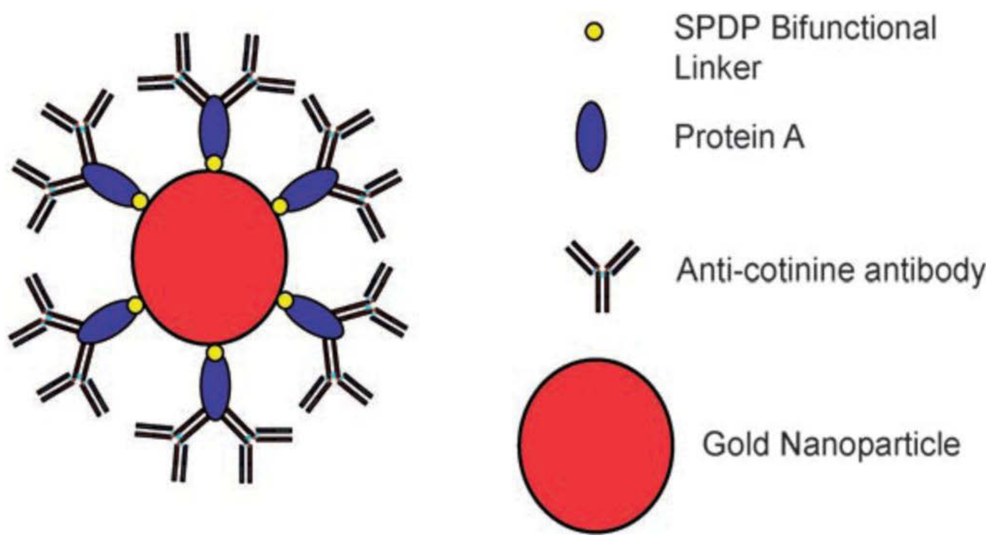


Figure 1-13: Schematic of the antibody-nanoparticle conjugate. The nanoparticles are linked by a bifunctional linker and protein A, which work as a biological linker to orientate the antibodies in the correct manner to the nanoparticle. Taken and reproduced from Leggett *et al.* [124].

The resultant antibody-nanoparticle conjugates were pipetted onto fingermarks (collected on glass slides) from smokers and left to incubate for 10 minutes. Any unbound antibody conjugates were removed by washing. Visualisation of the fingermark was carried out by the use of a secondary fluorescently tagged antibody fragment that binds only to the anti-cotinine antibodies [124]. Developed fingermarks were found to be of high quality, containing level 1, 2 and 3 detail (Figure 1-14). Control fingermarks, those of non-smokers, were found to have no fluorescence showing that no binding occurred through the anti-cotinine antibodies.

In further work, Russell and co-workers modified the conjugation approach by replacing AuNPs with magnetic nanoparticles. By doing this, the application and removal of the antibody conjugates becomes far easier and makes the technique exceptionally versatile [125]. Recombinant fusion protein A/G was used instead of protein A as the linker between the antibody and the magnetic particles. Using this method, the team targeted a range of drug metabolites: Δ^9-tetrahydrocannabinol (THC), methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) [125], cotinine [126], morphine, and benzoylecgonine [127]. In all of these studies, fingermarks from volunteers were placed on glass slides; the antibody-magnetic particle conjugate solution was then pipetted onto the fingermarks and left to

incubate for between 10 and 30 minutes at 37 °C in a wet chamber [126]. After incubation, excess antibody conjugate was removed by use of a magnetic wand. A secondary antibody fragment tagged with either Alexa Fluor 488 or Alexa Fluor 546 was then applied to the fingermark and left to incubate for a further 30 minutes before excess antibody was removed by washing twice [126]. Fingermarks developed via the targeting of each metabolite were classed as being of high evidential value and with tertiary detail visible [125-127].

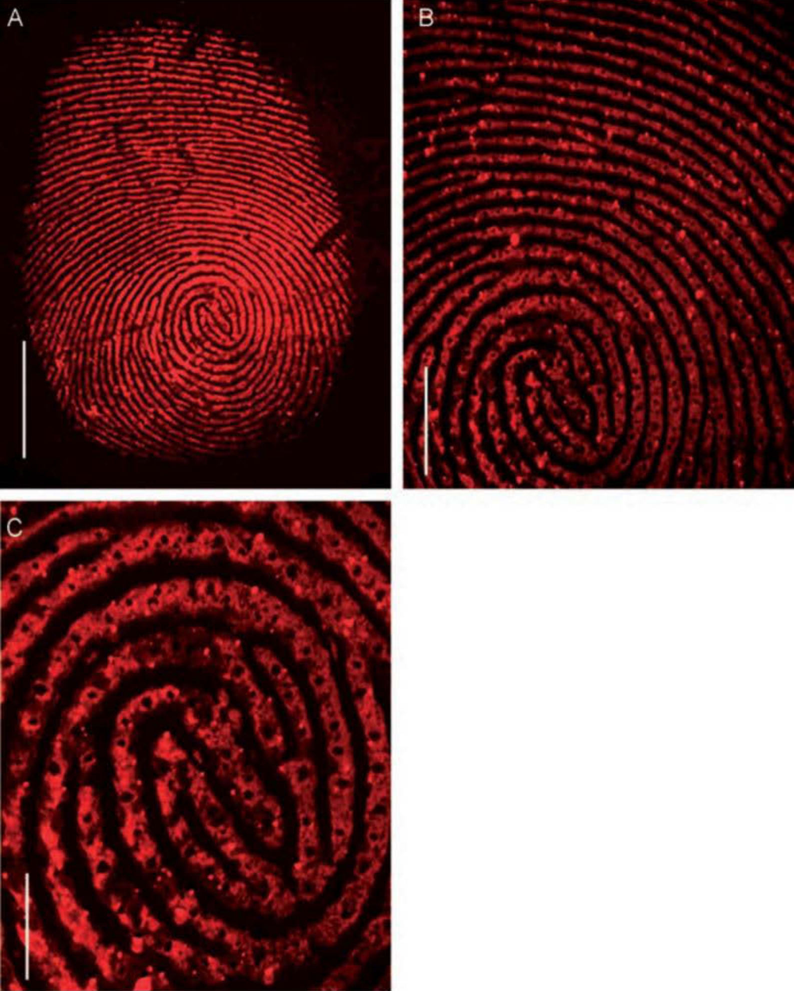


Figure 1-14: Fingermark of a smoker viewed under fluorescence using antibody-functionalised nanoparticles with secondary Alexa Fluor 546-tagged antibody fragment. Scale bar: 5 mm (A), 2 mm (B), 1 mm (C). Taken and reproduced from Leggett *et al.* [124].

The research carried out by the Russell group has shown great promise as a technique to both develop latent fingermarks and gain "extra information" in terms of drug use. However, the targeting of drug metabolites limits the use of this approach as a 'general' technique as such compounds are not universal in latent fingermark deposits.

A modified Western blot method was used to successfully detect latent fingermarks on porous substrates by Reinholz in 2008. The plasma protein albumin was selected as the target constituent as 60% of serum-protein volume is albumin, which is also secreted by the eccrine sweat glands [128]. Six porous materials including white envelope, recycled paper and nitrocellulose were used as test substrates. Marks were placed onto the substrates before being left to incubate for 30 minutes in a Petri dish containing Uni-Block solution (a commercial solution of tris-buffered saline, non-fat milk and Tween-20 solution). Rabbit anti-human whole serum was added to the Petri dish and left to incubate for a further hour. Following rinsing with a mixture of Tween 20 and tris-buffered saline (TTBS), blocking solution was again added along with goat anti-rabbit IgG and alkaline phosphatase and incubated for another hour. After further rinsing with TTBS, a staining buffer, along with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine saly was used to develop the marks. Development time was approximately 30 minutes depending on the surface and the development intensity required [128]. Marks were washed with deionised water and left to dry before photography of any developed marks.

Using this method, Reinholz was able to detect and develop marks on all substrate types with clear detail, including tertiary level features, on marks that were aged naturally for up to 130 days (Figure 1-15). The technique was also examined for use in sequence with a number of widely used porous development techniques. When used after DFO treatment, the albumin technique developed more detail than DFO on its own. When used after the development of marks with ninhydrin, the albumin technique removed the ninhydrin developed marks and failed to produce any ridge detail [128]. Using the technique after the development of marks with physical developer resulted in no improvement. Reinholz states that the albumin technique is as affordable as most currently used techniques, while the reagents needed are easily obtainable, are non-flammable and safe to use. This initial work clearly indicated that less commonly used fingermark constituents such as albumin can be targeted by an immunogenic reagent to successfully develop fingermarks with great sensitivity and selectivity.

Figure 1-15: A fresh fingermark on nitrocellulose substrate developed using the anti-albumin development technique. Taken and reproduced from Reinholz [128].

As antibodies have been found to be able to detect proteins in quantities ranging from 1 ng to 10 pg, Drapel *et al.* investigated the idea of using an antibody approach to detect and visualise latent fingermarks by targeting certain skin proteins [38]. Following preliminary research into the protein composition of epidermal secretions, they selected three of the most abundant proteins involved in the desquamation process during skin regeneration. Keratins 1 and 10, cathepsin-D and dermcidin were all chosen as potential antigenic components due to their various roles in skin regeneration and eccrine perspiration. Latent fingermarks were placed on polyvinylidene fluoride (PVDF) membranes and standard whitened and non-whitened paper before being treated with antibodies against each of the proteins selected using a standard immunodetection protocol (WesternBreeze® Chromogenic Kit from Life Science).

With this technique, fingermarks were detected on all substrate types with all three antibodies as seen in Figure 1-16. PVDF did produce better developed fingermarks than the paper substrates mainly due to the diffusion of the fingermark residues through the paper matrix. Both eccrine and charged fingermarks were detected, although charged fingermarks were better developed due to the loading of 'extra' proteins through the touching of the face [38]. Although proteins are not a common target for latent fingermark detection, all three

antibodies were able to develop fingermarks with tertiary level detail clearly visible (Figure 1-16). It was also noted that the antigens from the desquamation process (keratins 1 and 10; cathepsin-D) produced marks with very precise ridge edges, while the antigens from the sweat (cathepsin-D; dermcidin) produced marks with strong dotted patterns that can be attributed to the pores of the skin [38]. No comparison was made with commonly used techniques, but the sensitivity and selectivity of a protein targeting immunogenic reagent has been clearly demonstrated in this work.

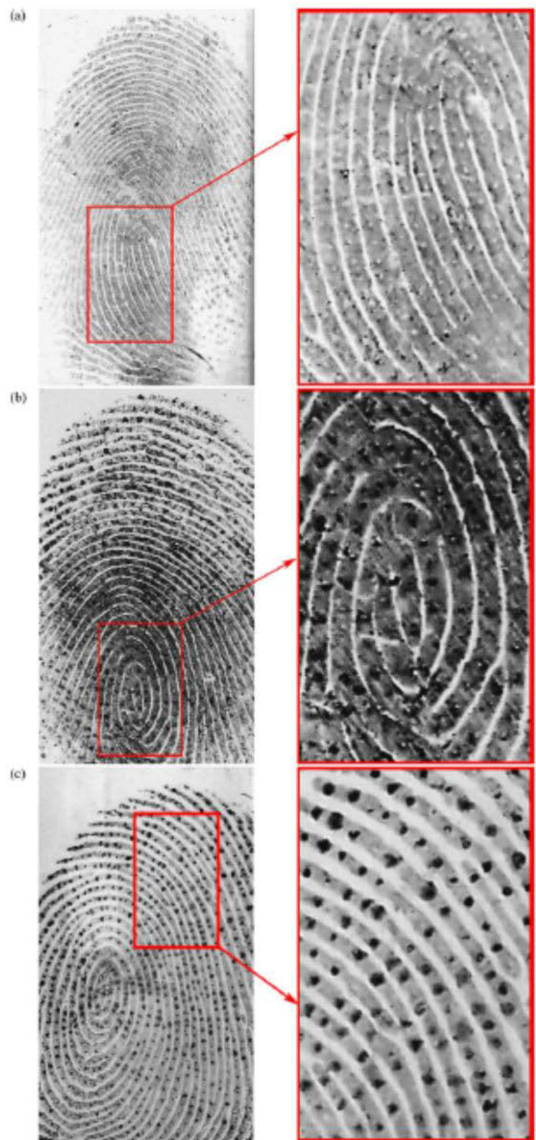


Figure 1-16: Detection of latent fingermarks on PVDF with: a) anti-keratin 1/10; b) anti-cathepsin-D; and c) anti-dermcidin antibodies. Taken and reproduced from Drapel *et al.* [38].

In work by Spindler *et al*. the use of antibodies conjugated to AuNPs was examined as an immunogenic technique targeting amino acids within latent fingermarks [129]. In this work, anti-L-amino acid antibodies were conjugated to 16 nm diameter AuNPs by either direct electrostatic adsorption or through covalent amide bond formation to a thioether [129]. These conjugates were dispersed in a water-acetone mixture before being applied to latent fingermarks placed on nonporous substrates. The antibody-nanoparticle solution was left to incubate at 37 ^{o}C for 30 minutes before excess unbound particles were washed off using acetone. In order to visualise the treated fingermarks, a secondary anti-rabbit antibody labelled with a fluorophore (Fluorescent Red 610 reactive) was added and left to incubate for 15 minutes at room temperature before being washed again (Figure 1-17). Developed fingermarks were visualised under fluorescence at 590 nm and observed using a 650 nm band-pass filter [129].

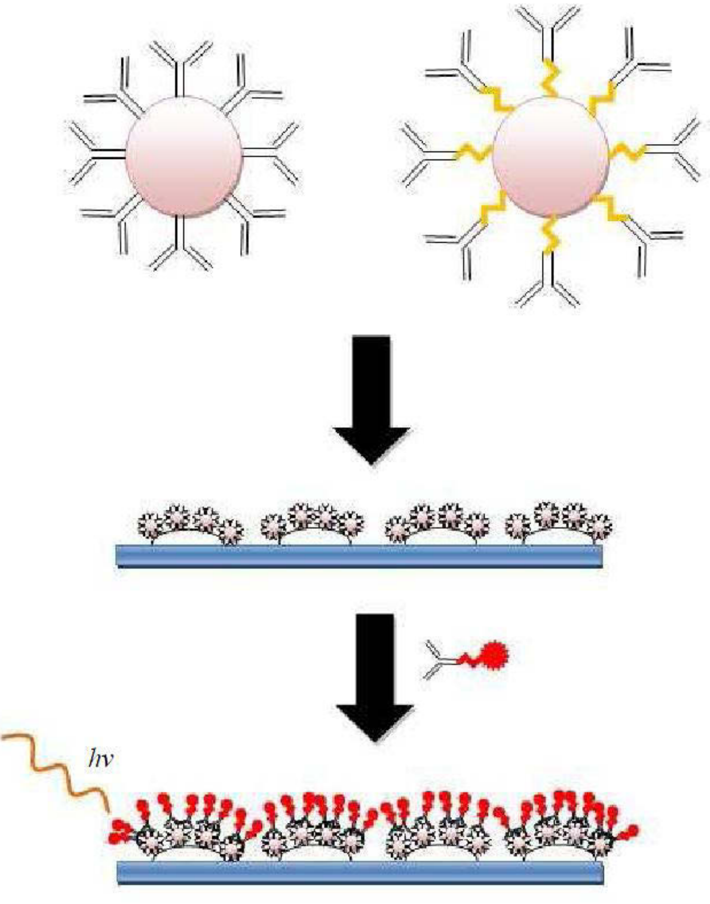


Figure 1-17: A schematic of the anti-L amino acid antibody-nanoparticle reagent. Taken and reproduced from Spindler [60].

Results showed that latent fingermarks could be detected and visualised on nonporous substrates using the anti-L-amino acid antibody approach (Figure 1-18). When compared to two commonly used nonporous techniques, cyanoacrylate fuming and dry powdering, marks developed with the antibody approach were found to be of less quality when dealing with fresh marks, particularly from highly eccrine donors. The reasons for this were believed to be due to fingermark residue dispersion caused by the solvent used for the immunogenic reagent and issues with background staining due to the low solubility of the antibody-nanoparticle conjugates within the carrier solvent [129]. However, when applied on marks that had been aged several months, the immunogenic reagent was found to outperform the commonly used techniques, especially dry powder. It was also found that the conjugation of the antibody to the nanoparticles was the key to successfully developing latent fingermarks. Comparing the reagent with an unconjugated anti-L-amino acid antibody reagent, the conjugated reagent provided significantly greater ridge clarity and fluorescence [129].

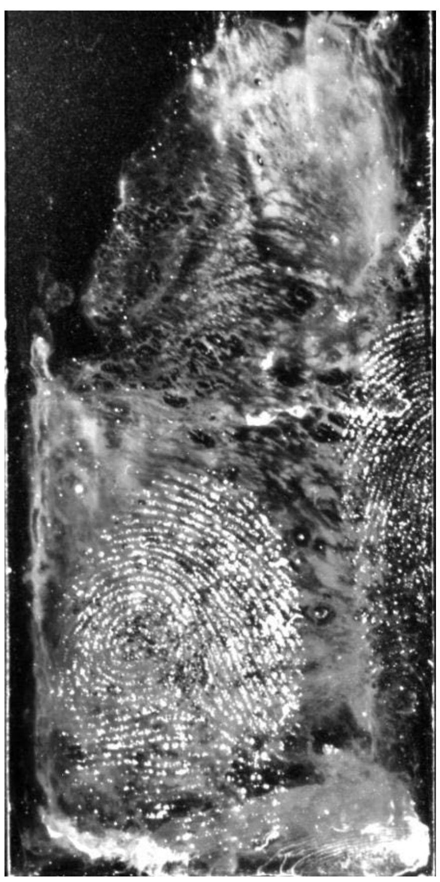


Figure 1-18: Fingermarks developed on glass slide: male donor-2 week old after secondary fluorescent tagging and post treatment wash with saline buffered solution. Taken and reproduced from Spindler [60].

Spindler and co-workers noted that an immunogenic approach for amino acids on nonporous substrates broadens the potential for detecting fingermarks on substrates that have usually been reserved for non-specific physical and physico-chemical detection techniques [129]. This may also result in more fingermarks being detected on these substrates, with the additional developed marks being those that current techniques may miss. With greater detection of aged and degraded marks than is possible with current methods, this research again clearly demonstrates the high sensitivity and selectivity of immunogenic reagents.

The use of antibodies as a novel technique to develop latent fingermarks has shown great promise in the quest for new detection techniques with improved sensitivity. With further antibody-fingermark research currently being undertaken, this approach could become a key detection technique, especially with aged and otherwise degraded fingermarks. Although this immunogenic approach has great potential as highly sensitive and selective fingermark reagents, there are, however, a number of issues that could limit its general use. These include the need for animal ethics clearance, antibody stability, production costs and various health and safety concerns.

1.2.2 Aptamers

As an alternative to antibodies, aptamers have been researched over the past 20 years. It is believed aptamers overcome the issues encountered with antibodies while still being able to be used under a number of different circumstances. Aptamers are short single-stranded nucleic acid oligonucleotides, either single-stranded deoxyribonucleic acids (ssDNA) or ribonucleic acids (RNA), which exhibit specific and complex three-dimensional stable structures [130]. These structures can be characterised by stems, loops, bulges, hairpins, pseudoknots, triplexes or quadruplexes. Through these three-dimensional structures, aptamers can successfully bind to a number of targets ranging from small molecular targets such as metal ions, organic dyes, amino acids, antibiotics, nucleotides and peptides, to complex mixtures and whole organisms including cell adhesion molecules, viral particles and pathogenic bacteria. Through various selection methods, aptamers can be selected to virtually any target with high sensitivity and selectivity, with binding affinities that can surpass those of monoclonal antibodies. An in depth discussion of aptamer science is covered in Chapter 2.

Detailed research into aptamers has allowed them to be used in many applications ranging from drug delivery molecules to highly specific and sensitive biosensors (see Section 2.5). Due to the many advantages of aptamers, including the high sensitivity and selectivity properties, aptamers are now rivalling antibodies in many common antibody approaches [131-133]. With the clear need for latent fingermark reagents that address the issues stated by several leading academics [3, 74, 75, 77], it stands to reason that aptamers should be investigated as a complementary immunogenic-based fingermark detection and visualisation technique aimed at achieving a transformational change in the sensitivity and selectivity properties of latent fingermark reagents.

1.3 Project aims and objectives

With the many theoretical advantages and possibilities of aptamer-based latent fingermark reagents, the ultimate aim of this project was to undertake an in-depth proof-of-concept study into the potential of aptamers as immunogenic-based latent fingermark detection and visualisation reagents. According to published research, this had not been previously attempted. Due to the uniqueness of this project, there were several aims and objectives that needed to be met in order to be able to provide a detailed investigation of the potential of aptamers in latent fingermark detection. These were as follows:

Aims

- To explore the use of aptamer technology for latent fingermark detection and enhancement;
- To develop aptamer-based reagents with high sensitivity and selectivity with the aim to overcome the issue of the non-negligible number of missed marks;
- To run comparison studies of the aptamer-based reagents against current, optimal techniques.

Objectives

- To investigate and determine the suitability of various aptamer sequences selected to latent fingermark components;
- To optimise the potential reagents for fingermark detection by:
    - Examining suitable solvent systems;

    - Exploring specific properties of potential visualisation molecules for compatibility;
    - Investigating any non-specific interactions;
- To examine and determine the binding of aptamers as a reagent to fingermark residue standards through the use of spot tests;
- To explore the role of substrates on aptamer interactions with fingermark residues;
- To run numerous fingermark studies to examine the reagents for selectivity and sensitivity;
- To compare the optimised reagents against standard reagents and techniques to determine the capacity of an aptamer-based reagent.

Due to the novelty of aptamers, it is first necessary to gain a comprehensive understanding of aptamers including their history, their selection and the current uses of aptamers. This background information is provided in Chapter 2. This chapter further provides support to split the overall project into two main areas of aptamer investigation, these being the use of RNA-based aptamers for the detection of amino acids and the use of DNA-based aptamers for the detection of the protein lysozyme. The investigation of RNA-based aptamers is the subject of Chapter 3. As this chapter describes the very first use of aptamers for the detection of latent fingermarks, many optimisations and investigations including reagent dissolution and substrate interactions are detailed, together with the detection of latent fingermarks on glass surfaces. Chapter 4 explores the investigation of DNA-based aptamers to lysozyme, a protein secreted by the eccrine glands. Chapter 4 focusses on the use of lysozyme aptamer reagents on PVDF and investigates both lysozyme and latent fingermark detection. Chapter 5 follows on from Chapter 4 and investigates the use of the lysozyme aptamer reagents for the detection of lysozyme and latent fingermarks on paper surfaces. This chapter also provides a comparison between the optimised aptamer reagents and the frequently used reagent IND-Zn.

Chapter 2: Aptamers

Chapter 2: Aptamers

2.1 Aptamer introduction

Aptamers are chemically synthesised, sometimes modified, polymers of nucleic acids that have been selected *in vitro* to bind to a chosen target. Aptamers generally range in size from six to 40 KDa and are composed of either RNA, ssDNA or a combination of these with non-natural nucleotides [134]. Aptamers often bind to their chosen target with strong binding affinities, with dissociation constants (k_d) generally achieved in the low nanomolar range (upon formation of a 1:1 complex) [135]. Through specific and complex three-dimensional stable structures, aptamers bind to their designated target through either a single feature or a combination, these being structural compatibility, stacking of aromatic rings, electrostatic and van der Waals interactions, and hydrogen bonding [136] (Figure 2-1). Aptamers have a high selectivity threshold, being able to distinguish between enantiomers of small molecules and even minor sequence alterations of macromolecules [134]. The selection of aptamers stems from large complex pools of nucleic acids and are generated through combinatorial chemistry via iterative cycles of adsorption, recovery and amplification (see Section 2.3) mimicking the Darwinian principle of evolution [134, 137]. It is through the huge diversity achieved by this method that aptamers are able to possess such strong affinity and selectivity levels. This approach also allows aptamers to be produced that are able to bind to almost any target through the relative ease of 'engineering' aptamers to suit the chosen target. Aptamers have been used in many different applications, including the delivery of drugs within the body, analysis of nucleic acid-protein interactions, and for the detection of various substances in complex mixtures [138]. Aptamers hold a number of advantages over other approaches, such as antibodies, that include their simplicity in selection (not requiring an animal host), high reproducibility, ease of modification, and improved stability and shelf life [131].

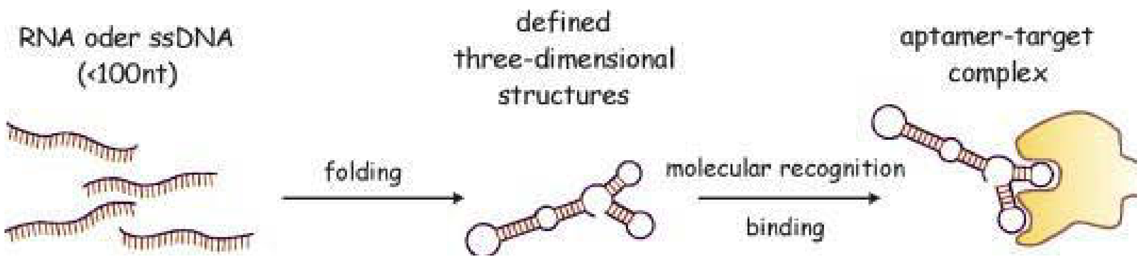


Figure 2-1: Schematic representation of the functionality of aptamers. Taken and reproduced from Stoltenburg *et al.* [130].

2.2 Aptamer history

Functional RNAs are known to possess both genotype (represented by a sequence that can be copied by a polymerase) and phenotype (represented by some functional trait that varies according to sequence) properties [139]. In the 1960s, Spiegelman and co-workers were the first to exploit this showing that Darwinian selection could exist in a cell-free system in his work with the RNA bacteriophage Qβ [140]. Spiegelman *et al.* found that this viral genome can be copied *in vitro* by the Qβ replicase protein. Wilson and Szostak neatly summarised this work as follows [139]:

> "Serial dilution experiments permitted hundreds of generations of genome replication to be performed quickly, with the phenotype under selective pressure being replication speed. Because of the inherent mutation rate of this polymerase, genotypic variation is generated *in vitro* during exponential genome amplification. The result of applying selective pressure to a population of variants was evolution, where the Qβ genomes adapted by deleting sequences unnecessary for recognition by the polymerase, thus shortening replication time".

In this research, Spiegelman also found that RNA sequences could be evolved to adapt to different niches such as the presence of ethidium bromide or an unbalanced composition of nucleotide triphosphates [140].

Although the work by Spiegelman above mainly investigated the templating functions of RNA, it also suggested the ability of RNA to fold into complex three-dimensional shapes and perform biochemical activities [141]. While these early *in vitro* experiments were very innovative, the fact that replication speed was the only phenotype that could be selected for

and that genetic variation was limited by the error rate of the polymerase caused limitations with his work [139]. Over 20 years later, with the idea of being able to bind to a target as an additional phenotypic selection on populations of RNA molecules, target binding sequences were selected. The ability of creating huge libraries of oligos with regions of completely random sequences through the chemical synthesis of DNA oligos, together with the advent of the polymerase chain reaction (PCR), allowed more practical ways of using *in vitro* selection and directed evolution to select new functional RNA oligos [139].

With these scientific advances, three separate research groups reported within the space of a year on the isolation of small nucleic acids that had predefined functions due to the experimental set-ups [142]. First, the Joyce group described the application of *in vitro* evolution to adapt the group 1 ribozyme so that it cleaved DNA rather than RNA [143]. Although this group started with a natural RNA-cleaving ribozyme rather than a complete random library, and were looking for a novel enzymatic activity rather than a selective ligand, their research did describe most of the steps used in aptamer selection [134].

Second, the Gold group designed an experiment to identify the sequence requirements of T4 DNA polymerase [144]. In this work, the library was based on the natural target hairpin structure but with the eight loop nucleotides randomised. Under numerous rounds of selection and amplification, the natural target of the enzyme was found to be the predominant sequence with high affinity. A second sequence was found with similar affinity but was a major variant to the other sequence. The Gold group were responsible for the naming of the *in vitro* selection method, referring to it as the Systematic Evolution of Ligands by Exponential enrichment or SELEX [144].

Within a month of the publication by the Gold group, the Szostak group described the use of *in vitro* selection to isolate molecules that demonstrated specific ligand-binding activities [145]. In a major difference to the previous two groups, Szostak *et al*. used a library that was completely structurally unrelated to any known nucleic acid, with nucleotides containing randomised regions of 100 nucleotides [134]. In a further difference, organic dyes were chosen as targets as they had no previously identified nucleic acid ligands. With the successful selection of binding sequences, the group named these nucleic acid ligands ‘aptamers’ deriving the word from the Latin expression “aptus”- meaning to fit and the Greek word “meros”-meaning part [145]. In the first 16 years since the publication of these three articles, there have been over 2000 articles appearing in the literature regarding aptamers

and their uses [146]. Interestingly none of them reported fingermark detection as a potential application of aptamers.

2.3 Systematic evolution of ligands by exponential enrichment (SELEX)

2.3.1 General principle

Since the introduction of SELEX by the Gold group, the process has generally remained the same, with the screening of very large combinatorial libraries of oligonucleotides in an iterative process of *in vitro* selection and amplification. In the early selection protocols, the targeting of proteins was very slow and cumbersome, with successful selections often taking several months [142]. However, selection protocols used today often identify aptamers within several days with the use of elaborate handling protocols including selections carried out with single beads, capillary electrophoresis (CE), surface plasmon resonance (SPR), high performance liquid chromatography (HPLC) and automated processes [142].

Although aptamers can be developed for a wide range of target molecules, as mentioned previously, there are prerequisites for potential targets to be successfully selected by aptamers with high affinity and selectivity. Single target molecules should be present in sufficient amount and with high purity to increase the specificity of selection by minimizing unspecific enrichment of binding oligonucleotides [130]. Aptamers bind to the target molecules by a number of intermolecular interactions including stacking interactions between aromatic compounds and the nucleobases of the aptamers, electrostatic interactions between charged groups or hydrogen bondings, and by the combination of complementarity in shape. Therefore, targets with positively charged groups, the presence of hydrogen bond donors and acceptors, and aromatic compounds make for more suitable targets. Aptamer selection is more difficult, but not impossible with negatively charged molecules and hydrophobic targets [130].

The starting point of any SELEX experiment is with the synthesis of a single-stranded library of oligonucleotides, each comprising a 5′- and a 3′- region of defined sequence but with a central random sequence region [147]. This random region is generally composed of between 20 to 80 nucleotides while the 5′- and 3′- regions are composed of 18 to 21 nucleotides [130].

The defined 5´- and 3´- ends enable the amplification of the selected oligonucleotides by PCR in each round of SELEX. A typical library contains between 10^{13} – 10^{15} different oligonucleotides; this huge complexity of the pool can be clearly illustrated when compared with the number of antibodies a mouse can generate, which is between 10^{9} – 10^{11} [148].

For the selection of DNA aptamers, the library can be used directly in the first round of SELEX (Figure 2-2). If RNA aptamers are to be selected, the DNA pool must first be transformed into an RNA pool. With the use of a special sense primer with an extension at the 5´-end containing the T7 promoter sequence and an antisense primer, the ssDNA library can be transformed into a double stranded DNA (dsDNA) library through PCR before being transcribed *in vitro* by T7 RNA polymerase [130]. Although the random sequence library can be used directly in the first round of SELEX, it is often found that the ssDNA/RNA sequence is amplified by PCR prior to the initial round of SELEX. Amplification is preferred prior to SELEX, as large scale amplification eliminates any damaged DNA synthesis products as these cannot be amplified by PCR. In either ssDNA or RNA, sequences will adopt various three-dimensional structures as a result of Watson-Crick base pairing and non-canonical intermolecular interactions [147].

The next stage in the SELEX process is the selection step and this includes the incubation of the pool with the target (binding), the subsequent partitioning of all unbound sequences, and then the elution of the bound oligonucleotides. This step is necessary to identify the selected molecules from the large variety in the oligonucleotide library that show the greatest affinity and specificity for the target molecule [130]. The library in a buffer of choice is incubated with the target molecule under a given temperature for a period of time. During this process, a small amount of the random sequence molecules interact with the target by adopting conformations that allow them to bind to the specific target [149]. A crucial moment in order to select aptamers of high specificity and affinity in the SELEX process is to efficiently partition the target-binding oligonucleotides from the non-binding [130].

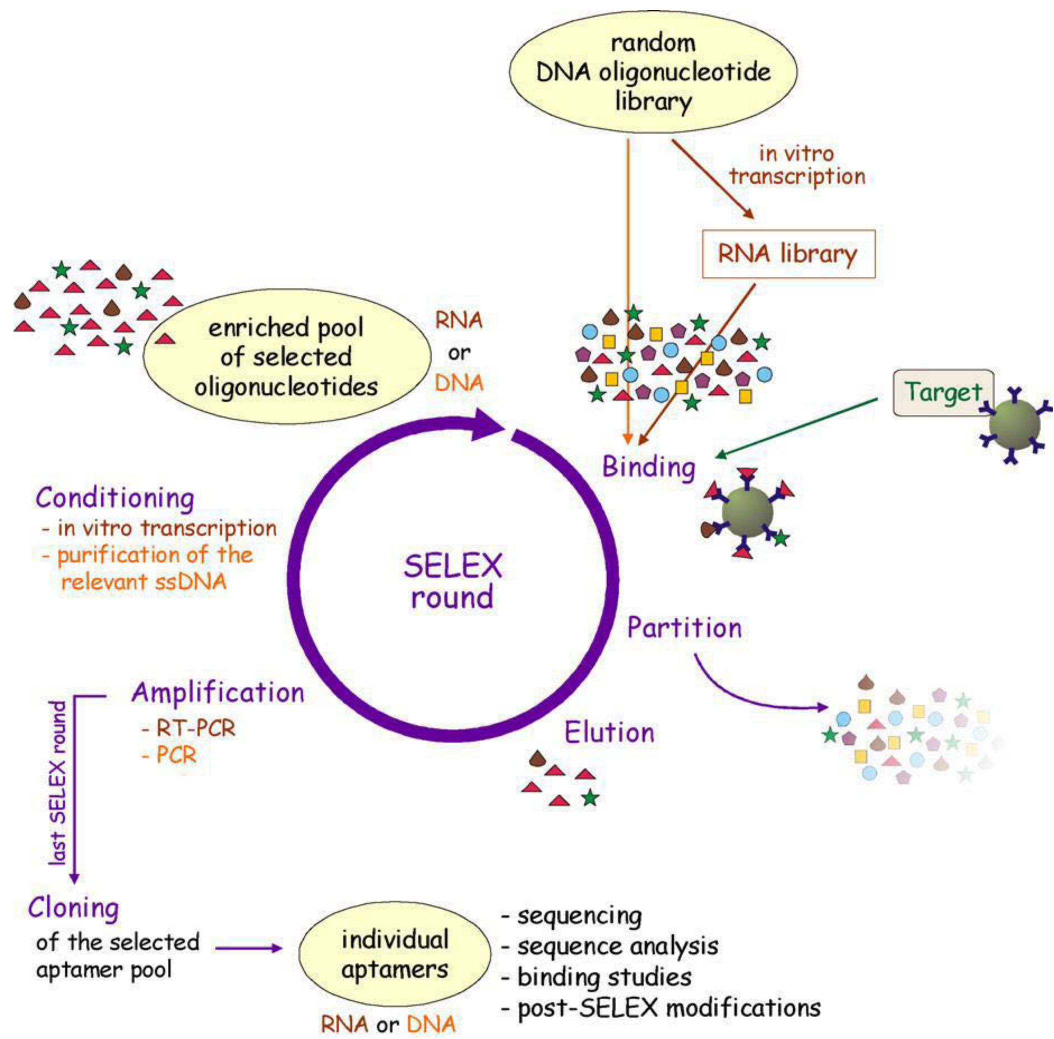


Figure 2-2: Schematic of the SELEX process for the *in vitro* selection of aptamers. Taken and reproduced from Stoltenburg *et al.* [130].

Partitioning can be done in a number of ways depending on how the target molecule has been interacted with the library. When in free solution, ultrafiltration by nitrocellulose filters of distinct molecular cut-offs is the most commonly used technique [130]. The use of nitrocellulose, however, can cause the loss of target binding oligos and also cause unspecific interactions of oligos with the membrane. The technique is used mainly for RNA aptamers with its efficiency for separating RNA from its protein complexes, however some DNA aptamers have been selected using this technique [130].

When the target molecule has been immobilised, the standard method for small molecular targets is by affinity chromatography for separation with column materials such as sepharose or agarose [150]. Although regularly used, affinity chromatography requires substantial amounts of target to achieve an efficient loading of the column [130]. With this, many other

techniques have been developed for the partitioning step, a few of these will be described here. The use of magnetic beads with the target attached before being incubated in the oligo pool has proven suitable for the selection of RNA aptamers. The use of magnetic beads allows for the easy separation of bound and unbound sequences [151]. UV cross-linking has been shown to be a suitable method for the selection of aptamers with high affinity. To do this, a photoreactive chromophore is substituted into the RNA sequence. When the target is in the presence of the pool, a UV monochromatic light is used to create a target-oligo complex; the complex can be partitioned further with nitrocellulose [152]. Gel electrophoresis can separate targets from samples based on the migration of molecules due to an electric field. The selected RNA is recovered from the gel by a crush and soak method before carrying out reverse transcription-PCR (RT-PCR). In some cases, this method has produced an increase of 20-fold in activity towards the target [153].

Surface plasmon resonance (SPR) has a number of advantages over the above methods. SPR is an evanescent wave biosensor technology that can monitor the interactions of two or more molecules in real time, which gives information on binding efficiency in the selection step. The pool is injected into the flow cell, a CM4 chip coated with the target, for two minutes. After, the binding buffer is injected into the cell at flow rate of 20 µL/min. The buffer is allowed to flow into a fraction tray and the collected fractions precipitated to recover bound RNAs for amplification before the next cycle [150].

One final technique is capillary electrophoresis (CE SELEX), which has several advantages over other techniques including speed, resolution, capacity, minimal sample dilution and it requires less cycles than other techniques [154, 155]. The target is first incubated in a free solution with the random pool. It is then applied in nanolitre volumes to a capillary at various pressures for a number of seconds. Using high voltage, the solution is separated, the bound fraction is separated from the unbound due to charge and size, and the bound molecules amplified and purified before undergoing another selection round (Figure 2-3). No matter what partitioning technique is used, the partitioning criteria are initially set so that a large amount of the nucleic acids is retained during separation (generally between 5 – 50%). This is due to the fact that only an extremely low number of sequences with the highest affinity nucleic acids exist in the oligonucleotide library [156].

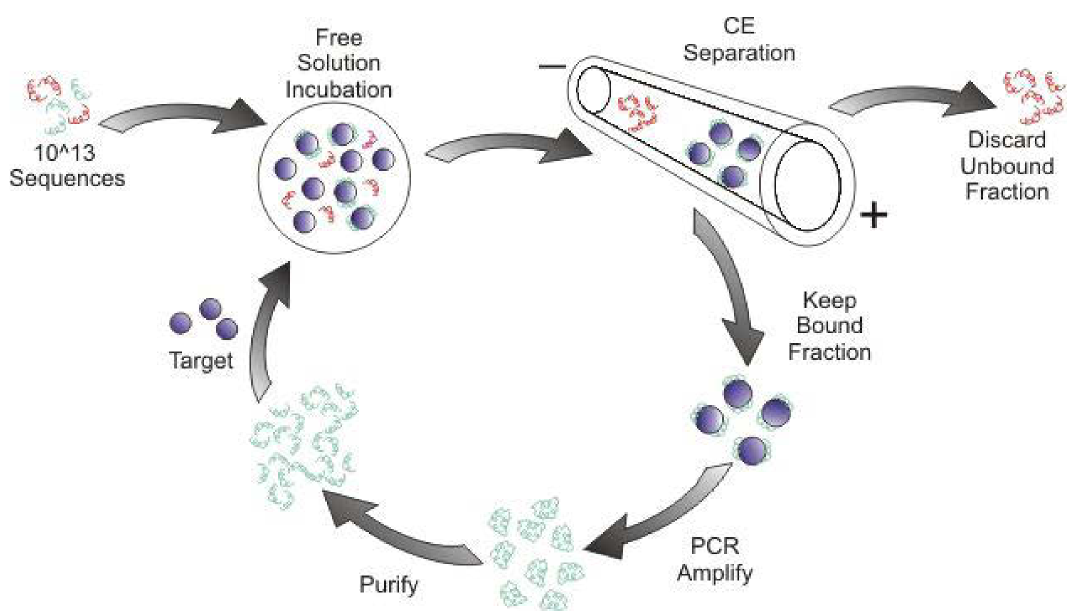


Figure 2-3: Schematic of the CE SELEX method showing the separation of bound and unbound sequences through the use of a capillary. Taken and reproduced from Mosing [154].

Once non-bound and bound sequences have been partitioned, the bound sequences usually need to be eluted from the target. This can be done by denaturing methods such as heat treatment, high pH and low salt concentrations, or by the addition of substances, such as the surfactant sodium dodecyl sulfate (SDS), or the chelating agent ethylenediaminetetraacetic acid (EDTA). Affinity elution by use of target and elution with competitive binders can also be used. Photoelution, where photocleavable linkers are used is also possible [130].

After each selection step, the resulting oligonucleotides showing affinity for the target are amplified. This is because only a few functional oligonucleotides will be selected due to the high complexity of the initial pool and, as the process continues, the pool needs to be amplified to contain only the sequences with the highest affinity for the target. Amplification is carried out by PCR; however, it depends on the type of oligonucleotide. For RNA-based aptamers, the selected pool has to be first run through RT-PCR, which results in the corresponding cDNA being achieved which is then amplified by PCR [130].

After amplification, the pool needs to be conditioned to prepare it for the next round of selection. After undertaking PCR of the bound oligonucleotides, the pool is in the form of dsDNA. For RNA pools, transcription with T7 RNA polymerase is necessary. For DNA pools, the dsDNA has to be separated to create ssDNA. However, as only the sequence that shows affinity for the target molecule is wanted, the DNA needs to be separated so that the

corresponding strand can be removed from the pool leaving just the ssDNA sequence from the selection round. Separation can be carried out in a number of ways. Using biotin/streptavidin allows for successful separation by adding biotin to the unwanted strand and using the size difference in gel electrophoresis to distinguish between strands, while streptavidin can be allowed to bind dsDNA to surfaces to allow separation after denaturation [130]. Biotinylated reverse primers can also be used during PCR amplification and strands made single by running them through a streptavidin-agarose column allowing the wanted ssDNA to be recovered [157]. Other methods include separation of fluorescently tagged ssDNA by denaturing polyacrylamide gel electrophoresis (PAGE), where the relevant DNA strands can be found in the gel by using a UV transilluminator; the corresponding DNA bands can then be cut out of the gel and the DNA eluted ready for the next round of selection [158].

The number of rounds needed to select suitable aptamers with high affinity for the target is dependent on the diversity of the library, affinity towards the target molecule, stringency of selection and bias for amplification. Usually 8-15 rounds are needed until the 'winning' aptamer is found (where the pool of DNA/RNA is dominated by those sequences that bind to the target best). Using fluorescent labelling, the quantification of the SELEX procedure after each cycle can be done without the addition of further chemicals. The DNA/RNA in the different fractions (binding solution, unbound solution, washing solution and DNA/RNA from elution) of each round is measured by fluorescence and quantified by using a calibration plot [158]. An alternative method of quantification can be done using affinity capillary electrophoresis. After each cycle, a small amount of the pool (0.5 µL aliquot) is PCR amplified for five cycles in the presence of a fluorescent primer to fluorescently tag the sequences. The tagged pool is incubated with increasing concentrations of the target and analysed by CE. The peak height of the free aptamer can be fitted to an equation to estimate the dissociation constant [155].

Once the final round of SELEX has been completed, the remaining pool of sequences is amplified before the nucleotide sequences of the individual molecules are determined. To do this, the final pool is cloned into a bacterial vector and individual colonies are sequenced. Normally 50 or more aptamer clones are found and analysed by sequencing and sequence analysis. Sequence alignments are very useful to determine the complexity of the aptamer pool and in the identification of aptamers with homologous sequences [130]. Binding studies for the determination of specificity and affinity of the selected aptamers are usually carried out. They are an important part of aptamer selection as applications using aptamers depend

on the exact ascertainment of specificity and affinity. The affinity of an aptamer is measured by the dissociation constant, K_d. Once the sequence of the aptamer has been determined, the aptamer can be chemically synthesised [149].

2.3.2 SELEX modifications

One of the main advantages of aptamers and the SELEX protocol is the wide variety of modifications that can be undertaken at many different stages during their selection. These modifications can be carried out both before and after the selection cycle and also during SELEX. Modifications can be undertaken to improve the characteristics of the aptamers for a given application and can include improving the stability of the aptamer, optimising the binding parameters, and undertaking negative selection cycles to remove un-wanted aptamer sequences. Some modifications available for SELEX are shown in Table 2-1 together with a description of the benefits of these variations. For a concise review of the modifications, the interested reader is referred to the review by Stoltenburg *et al.* [130].

Prior to the SELEX process, the starting oligonucleotide library can be chemically altered by a number of modifications. In one example, the modification of uridine triphosphate (UTP) allows the attachment of a variety of substituents to the 5-position of UTP which are compatible with the enzymology of the SELEX process [159]. Incorporation of groups including aromatic, alkyl, heterocycle and amino acids into modified starting libraries allows the fine-tuning of electrostatic and hydrophobic interactions between the nucleic acids and their targets [159]. Other modifications prior to SELEX include modifying the phosphate backbone of nucleic acids. Replacement of the oxygen in the phosphodiester linkage by sulfur produces a phosphorothioate linkage that increases the resistance to nuclease digestion [130].

Table 2-1: A selection of the modifications available for SELEX. Adapted from Stoltenburg *et al.* [130].

SELEX Modification	Description of modification/Benefit
Negative SELEX	Minimises the potential of co-selection of unwanted aptamers by undertaking pre-selection with molecules that should not be recognised and removal of these sequences from the pool
Counter SELEX	Produces aptamers that are able to discriminate between closely related structures through the introduction of a related target to the pool to remove sequences that are not able to distinguish between the related targets
Blended SELEX	Enlarges the nucleic acid molecules by the use of special non-nucleic acid components giving aptamers additional properties other than binding abilities
Covalent SELEX	Utilises aptamers that contain a reactive group capable of covalent linking to a protein target
Photo SELEX	Uses aptamers that contain photoreactive groups allowing the aptamers to bind and photo cross-link to a target or photo activate the target molecule
Tailored SELEX	Useful for selecting short aptamers by identifying aptamers with only 10 fixed nucleotides through ligation and removal of primer binding sites within SELEX
Signalling aptamers	Aptamers can report binding through the switching of their structure to show a signal such as fluorescence
Toggle SELEX	Able to switch between different targets during alternating selection steps
FluMag SELEX	Oligonucleotides contain a fluorescent modification while the target is immobilised on magnetic beads

Post-SELEX modifications can be made to the bases and/or riboses of the aptamer. The single sequence site substitution of a modified thymidine base to an aptamer to basic fibroblast growth factor showed increased affinity [159]. Two substitutions were examined to a position of low sequence conservation surrounded by highly conserved residues in a tetraloop. The modified bases 5-[*N*-(aminoethyl)-3-acrylamido]deoxyuridine and 5-[*N*-(aminoexyl)-3-acrylamido]deoxyuridine both improved aptamer affinity with an increase of over fivefold by

the latter [159]. Reselection of an aptamer or aptamer pool by subjecting the aptamer to a second selection process where the existing aptamer sequence is diversified by mutagenesis to create a new library can generally improve affinity or specificity [130]. Further modifications with functional groups can be carried out for immobilisation (biotin, amino groups) or detection (fluorescent molecules) [130]. Fluorescent molecules can be easily attached to aptamers in a number of approaches such as monochromophore, bischromophore, *in situ* labelling, dye-staining and aptamer-polymer conjugates [160]. Furthermore, various nanoparticle conjugations and quantum dot linked aptamers have been used with great success in sensitivity and visibility [161-163].

2.4 Comparison between aptamers and antibodies

Aptamers are a class of molecules that are now rivalling antibodies in both diagnostic and therapeutic techniques. While both can target and bind with high specificity and affinity, aptamers have several advantages over antibodies. One of the main advantages is on ethical grounds, as no animal is needed for the production of aptamers since they are identified through *in vitro* selection. Molecules that do not generate a good immune response or which are toxic in antibody production show none of the problems in aptamer selection, easily generating high affinity aptamers [131, 147]. In addition, as antibodies are produced by an animal's immune system, it is this that selects which sites on the target the antibodies bind to. This restricts identification of antibodies that can recognise targets to particular physiological conditions. Aptamers, on the other hand, are able to be manipulated to bind to specific regions of the target with specific binding properties under different binding conditions [147]. Due to the nucleic acid composition of aptamers, they exhibit very low to no immunogenicity, which is very useful for the use of aptamers within the human body. Antibodies, on the other hand, are significantly immunogenic, which precludes repeat dosing [164].

After the SELEX process, aptamers are chemically synthesised and purified to a high degree of accuracy. They can also be modified by the introduction of various functional groups to improve affinity, specificity, stability and other characteristics. Antibodies tend to suffer with batch-to-batch variations; aptamers, on the other hand, are able to give a very high degree of reproducibility as they are chemically synthesised [131, 147]. Another major issue with antibodies is that they are sensitive to temperature and undergo irreversible denaturation,

while also possessing a limited shelf life. Aptamers can also undergo denaturation; however, they are able to be easily regenerated within minutes back to their active conformation. They are also able to cope with long-term storage and are easily transported at ambient temperature [131].

Aptamers are easy to tag with reporter molecules for the detection of binding, such as with biotin and fluorescein, which can be precisely attached at locations on the aptamer as chosen by the user [131]. In order to produce monoclonal antibodies, the identification and production process is very labour intensive and can become very expensive in the search for rare antibodies that require the screening of large numbers of colonies [131]. Depending on the affinity required and choice of technology used for aptamer selection, production can be much quicker and easier. Table 2-2 summarises some of the advantages of aptamers when compared with antibodies.

Table 2-2: Summary of selected advantages of aptamers when compared with antibodies. Adapted from O'Sullivan [165].

Antibodies	Aptamers
Limitations against targets representing constituents of the body and toxic substances	Toxins as well as molecules that do not elicit good immune response can be used to generate high affinity aptamers
Kinetic parameters of Ab-Ag interactions cannot be changed on demand	Kinetic parameters such as on/off rates can be changed on demand
Antibodies have limited shelf life and are sensitive to temperature and may undergo denaturation	Denatured aptamers can be regenerated within minutes; aptamers are stable to long term storage and can be transported at ambient temperature
Identification of antibodies that recognise targets under conditions other than physiological is not feasible	Selection conditions can be manipulated to obtain aptamers with properties desirable for *in vitro* assays, e.g. non-physiological buffer
Antibodies often suffer from batch-to-batch variations	Aptamers are produced by chemical synthesis resulting in little or no batch-to-batch variations
Requires the use of animals	Aptamers are identified through an *in vitro* process not requiring animals
Labelling of antibodies can cause loss in affinity	Reporter molecules can be attached to aptamers at precise locations not involved in binding

2.5 Applications of aptamers

2.5.1 Therapeutic applications

The applications of aptamers within the field of science are wide ranging and growing rapidly as technology develops and the understanding of aptamers increases. As previously mentioned, aptamers can rival and, in some cases, improve on the features of antibodies. Therefore, aptamers are currently being researched and tested as replacements of many antibody-based techniques. One such area where aptamers hold great potential is with therapeutic applications. In this field, aptamers have been successfully used for various functions such as inhibitory aptamers, decoy-like aptamers, regulatable aptamers and multivalent aptamers [166].

Inhibitory aptamers are being heavily researched due to the capabilities of these aptamers in inhibiting various biological functions on targets including soluble proteins, transcription factors, cell surface receptors and other intercellular proteins [166]. One inhibitory aptamer that is now approved for use both in America and Europe is pentaptanib. This aptamer is used for the treatment of age-related macular degeneration (AMD) by targeting the heparin-binding domain of vascular endothelial growth factor (VEGF), which promotes the blood vessel formation related to AMD [167]. From this success, several other aptamers are currently under clinical development as highlighted in Table 2-3.

As decoy-like aptamers, the inhibiting of binding proteins such as HIV-tat, NF-$_{\kappa}$B and E2F to their cognate sequences on DNA has been shown to be possible. As DNA- or RNA-binding proteins have a natural tendency to bind with nucleic acids, aptamers can mimic the target sequence of the protein and thus act as a decoy and prevent transcription of the target gene [166]. Regulatable aptamers have been found to be of use in certain clinical applications where the rapid inactivation of a drug is necessary. The REG-1 aptamer (Table 2-3) works as a two-part therapeutic agent with an RNA aptamer (RB006) specific for the coagulation factor IXa and a complementary RNA oligonucleotide (RB007). When inhibition of factor IXa is required, the RNA aptamer is administered, but can be stopped by the antidote (RB007) [168]. Multivalent aptamers have been investigated in several studies where aptamers have been assembled in specific approaches that have seen increases in aptamer avidity, stability and effect [166].

Table 2-3: Various aptamers currently in clinical development. Adapted from Thiel and Giangrande [166].

Aptamer, target	Company	Disease(s)	Stage of clinical development
Pegaptanib, VEGF	Eyetech Pharmaceuticals/ Pfizer	AMD, diabetic retinopathy	FDA-approved AMD 2004; Phase II for diabetic retinopathy: improved vision, decreased edema
REG-1 (RB006/RB007), Factor IXa	Regado Biosciences	PCI, CABG	Phase I: Reversible anti-coagulation; Phase II trial completed
AS1411, Nucleolin	Antisoma	RCC, AML	Phase I: Tumour regression; Phase II underway
NU172, Thrombin	Nuvelo/Archemix	PCI, CABG	Phase I: No adverse safety profile; Phase II planned
ARC1779, von Willebrand factor	Archemix	TMA, TTP, CEA	Phase I: No adverse effects in healthy patients; Phase II underway
ARC183, Thrombin	Archemix	CABG	Phase I: Suboptimal dose profile; withdrawn

With the high selectivity and sensitivity of aptamers, together with their ability to penetrate cells due to their small size, aptamers are increasingly being utilised in drug delivery applications. The delivery of small interfering RNAs (siRNA), toxins, radioisotopes and chemotherapeutic agents encapsulated in nanoparticles have all been undertaken [166]. The most established aptamer for this method is a nucleic acid molecule that targets the prostate-specific membrane antigen (PSMA). The PSMA is a molecular antigen associated with the onset and progression of prostate cancer. Through modification of the aptamer with polymer-coated nanoparticles with encapsulated siRNAs, toxins and drugs, it is possible to target specific cancer cells with the anticipated unwanted side effects being significantly reduced [142] (Figure 2-4).

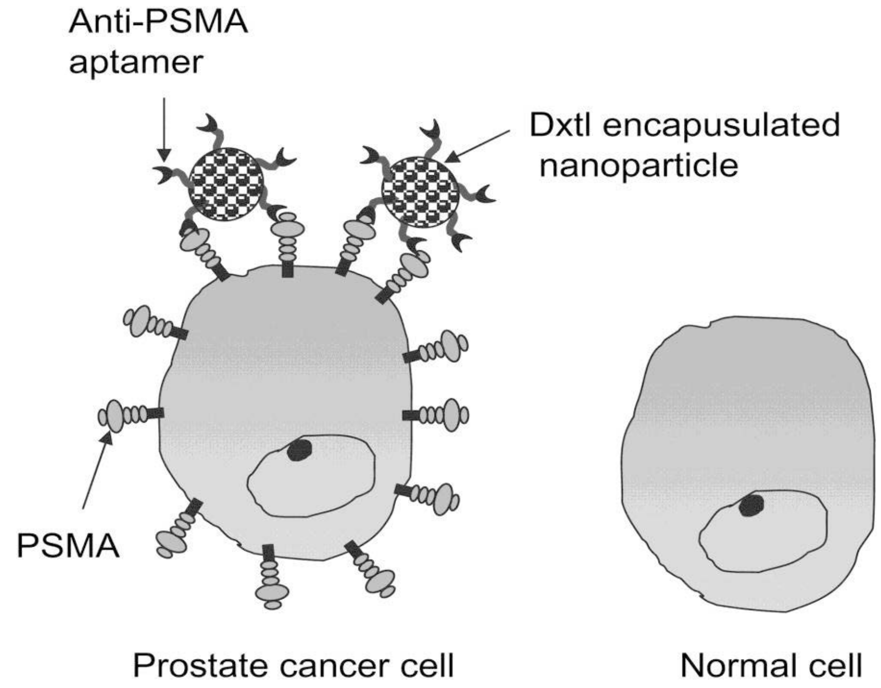


Figure 2-4: The use of aptamers as drug delivery molecules for drugs targeted at specific cells. Taken and reproduced from Khati [133].

2.5.2 Analytical and biosensor applications

2.5.2.1 Analytical

As well as their use in therapeutics, aptamers also have a wide range of possibilities in analytical chemistry. Aptamers are an ideal device for use in chromatography due to their high affinity to specific targets as well as their ease of modification and immobilisation, good stability and highly reproducible properties [169]. L-Selectin has been successfully purified from Chinese hamster ovary cells transfected to express the protein using affinity chromatography with an aptamer specific for the molecule [170]. The separation of adenosine monophosphate (AMP), nicotinamide adenine dinucleotide (NAD^+), cyclic-AMP, adenosine diphosphate (ADP), adenosine triphosphate (ATP) and adenosine was carried out by an aptamer specific for adenosine and ATP. After the addition of a biotin label and incubation with polystyrene porous particles or streptavidin porous glass beads, the resulting particles were placed into fused silica capillaries leading to affinity chromatography capillaries. These capillaries showed a 3.3 fold increase in aptamer density on the silica media compared to the density possible with IgG on similar media [171].

Capillary electrophoresis is another successful technique for affinity studies due to low sample and reagent consumption, short analysis times, high separation performances and ease of modification. In many CE assays, laser-induced fluorescence (LIF) detection is used due to the sensitivity and selectivity of detection [169]. As a large number of analytes in CE need to be derivatised for LIF, aptamers can provide a molecular basis for LIF so long as any binding can be represented through signal changes [169]. This has been proven in a number of successful detections [172-174]. As has already been shown, CE can be used in the SELEX cycle for the selection of aptamer sequences (see Section 2.3.1). In some cases, the use of CE has been able to select aptamer sequences in two rounds of SELEX, where standard SELEX generally requires between 8 – 15 rounds [175].

The use of aptamers as recognition elements in microfluidic devices has also been shown to have great potential as highlighted in a number of studies [176-178] (Figure 2-5). In one example, the use of aptamers immobilised in a microfluidic channel to capture rare cells as a rapid assay without any pretreatment has been possible with outstanding levels of enrichment purity (97%) and capture efficiency (80%) [179]. Aptamers have also been shown to be successful as substrates for protein capture and analysis in affinity MALDI MS. This was highlighted by using a thrombin targeted aptamer for the direct detection of thrombin after covalently bonding to a fused silica glass surface [180]. In this approach, the detection of thrombin down to 5 pmol was possible. In HPLC, aptamers have demonstrated their capability for stereoselectively binding to target chiral compounds, whereas alternatives such as imprinted polymers or antibodies have shown various drawbacks, such as affinity tags that can cause protein structure and function issues [149].

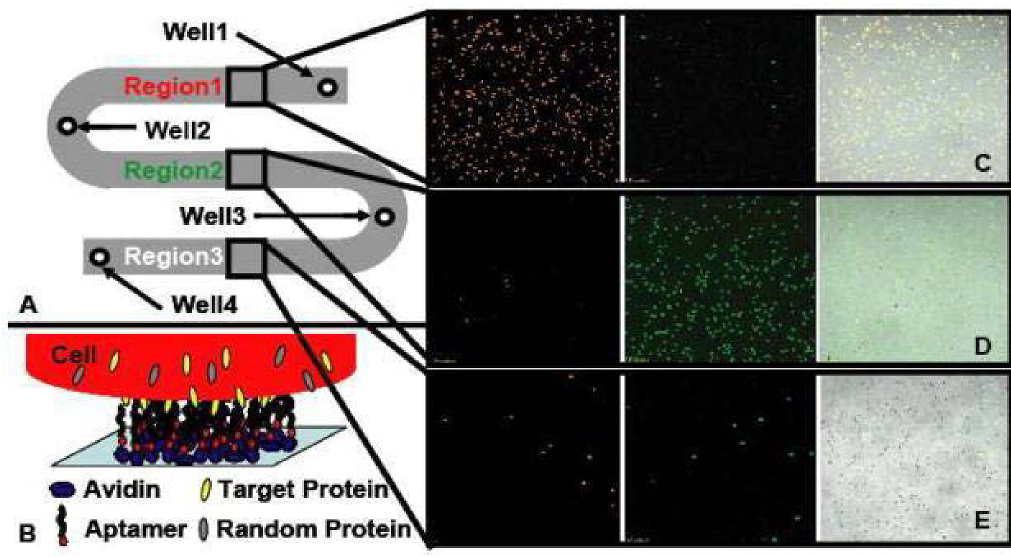


Figure 2-5: Schematic of the microfluidic device used to capture three different stained cells, highlighting three regions for aptamer immobilisation and four wells for channel preparation and cell sample injection (a). Avidin is adsorbed onto the glass surface immobilising biotinylated aptamers (b). Representative images showing the capture of three different stained cells (CEM cells stained red, Ramos cells stained green and Toledo cells unstained). Adapted from Xu *et al.* [176].

2.5.2.2 Biosensors

As biosensors rely on the power of a molecular recognition element, aptamers are ideally suited as a new type of element. Biosensors allow for the rapid detection and quantification of analytes by immobilising antibodies on sensor surfaces to allow them to come into contact with the analytes. However, the capacity for the regeneration of antibodies is poor and leads to the loss of activity of the surface immobilised antibodies [131]. The use of aptamers has several benefits with the main advantage being that aptamers can be easily regenerated using a number of methods. Biosensors that are based on aptamers are referred to as 'aptasensors' and can be constructed through a number of different methodologies including electrochemical, optical and mass-sensitive biosensors [164]. In general, aptasensors are based on DNA aptamers rather than RNA aptamers due to their greater stability and resistance to degradation by ribonucleases; however, some RNA methods are possible with various chemical modifications [169].

Many electrochemical aptasensors have been developed for the sensitive detection of a range of molecules due to the attractiveness of electrochemical analysis (high sensitivity, compatibility, inherent miniaturisation, low cost). Electrochemical aptasensors have been constructed using various techniques, including electrochemical impedance spectroscopy,

potentiometry with ion-selective electrodes, electrochemiluminescence (ECL), cyclic voltammetry and differential pulse voltammetry [164]. In one method, the detection of thrombin was possible through the immobilisation of a thrombin-binding aptamer onto multi-walled carbon nanotubes. This allowed the aptamer to be both the molecular recognition element and the carrier of the electrochemical capture probe [169].

As optical aptasensors, many different approaches have been used to provide optical detection including chemiluminescence, fluorescence, quantum dots and colorimetric strategies. Chemiluminescence detection using aptamers has been able to detect thrombin with a 26 fM detection limit through the aggregation of AuNPs in a luminal-H_2O_2 chemiluminescence reaction [181]. This method showed almost four orders of magnitude better sensitivity than any currently used AuNPs-based colorimetric methods [169]. Many fluorescence based aptasensors have shown detection limits at the subpicomolar level with the use of various fluorescence strategies including near-infrared and Alexa Fluor aptamer conjugations and the use of fluorescence resonance energy transfer (FRET) [182-184].

The benefit of mass sensitive detection is that mass changes can be recorded on the sensor surface without the need for additional labelling reagents while detection can be undertaken in real-time [169]. SPR is the choice of method for this approach, with several different methods of incorporating aptamer technology. Overall, the methods have provided better results than with typical SPR sensors [169].

The use of aptamers in clinical diagnostic tests has also shown great potential. Aptamer-linked immobilised sorbent assay (ALISA) or enzyme-linked oligonucleotide assay (ELONA) have proven their feasibility in comparison studies with the common antibody based ELISA technique. The main benefit of using aptamers over antibodies is the unlimited potential of aptamers to circumvent the limitations associated with antibodies [185]. Rapid diagnostic tests (RDT) are another common tool for the diagnosis of infectious diseases such as malaria and influenza [164]. Several aptamer-based RDTs for the detection of various biomarkers to diseases have recently been introduced. Through the modification of aptamers with functionalised AuNPs, specific RDTs have been produced to create dry-reagent strip aptasensors (Figure 2-6). In one study, the rapid, specific and sensitive detection of Ramos cells spiked into human blood was possible [186]. The visual detection of thrombin in human plasma has been achieved within minutes [187]. In the thrombin approach, sensitivity and specificity were found to be superior to those of the antibody-based strip sensor.

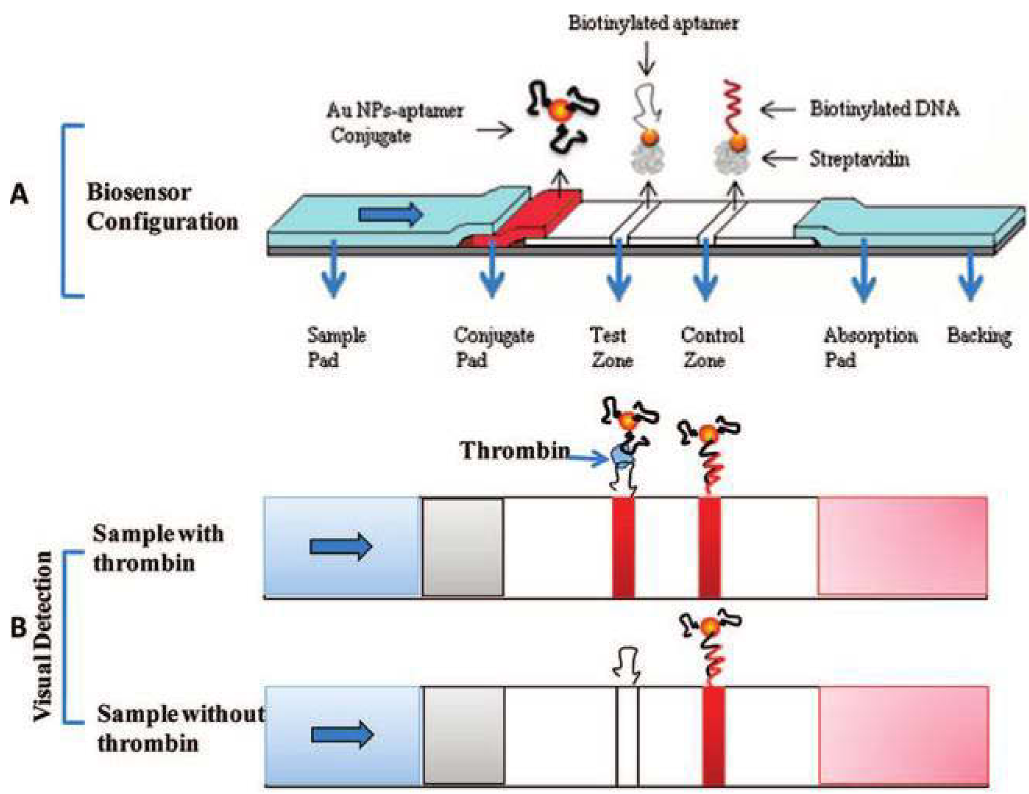


Figure 2-6: Schematic of the aptamer-AuNP based strip assay. Configuration of the RDT (a), representation of visual detection in the presence or absence of thrombin (b). Adapted from Xu *et al.* [187].

2.5.3 Current use in forensics

It would appear that aptamers have not yet been used operationally for any type of detection method within the field of forensic science. They have, however, been briefly investigated as possible methods for the detection of cocaine and methamphetamine as has been reported in three studies. By adaption of lateral flow devices with aptamers instead of antibodies, simple “dipsticks” or litmus test type assays were created. In the study by Liu *et al.,* nanoparticle aggregates were linked by a cocaine targeting aptamer [188]. These were directly added to the conjugation pads of a composite pad strip. By dipping the wicking pad section into human serum spiked with cocaine, the lateral flow device detected cocaine at concentrations of 0.2 mM and above. This study highlights that immobilised cocaine aptamer-linked nanoparticles have the ability to detect cocaine in complex matrixes such as human blood serum. In the second study, the use of quantum dots with functionalised surfaces for the attachment of aptamers targeted towards cocaine were used [161]. The aptamer-quantum dot method was shown to have several advantages over the more

traditional techniques including simple sample preparation, high sensitivity and extremely low sample consumption.

The selection of aptamers to methamphetamine was undertaken by Nilsen-Hamilton and Krau with the aim to develop a microarray for the screening of a variety of drugs to ‘provide forensic investigators with a rapid screening procedure for many common drugs’ [189]. Although it is believed that an aptamer to methamphetamine had been selected, unfortunately the project was not completed and a microarray system was not developed. This research, however, does highlight how aptamers could be used in an area of forensic science currently struggling to keep up with an ever changing illicit drug market.

As has been highlighted throughout this chapter, aptamers possess many characteristics that make them an ideal device for the high sensitivity and selectivity of target molecules. As described in Chapter 1, latent fingermark detection relies on the detection of certain molecules and compounds within a latent fingermark to enable visualisation. With the current issues surrounding fingermark detection techniques, including lack of sensitivity, it can clearly be seen that the properties aptamers possess could be of great benefit in the field of latent fingermark detection. This will be the subject of the following chapters.

Chapter 3: RNA aptamers to amino acids and fingermark detection

Chapter 3: RNA aptamers to amino acids and fingermark detection

3.1 Introduction

3.1.1 Amino acid suitability for latent fingermark detection

As mentioned previously (see Section 1.1.5), eccrine sweat contains amino acids that are secreted through the eccrine sweat glands found along the ridges of friction ridge skin. Hence, when a finger comes into contact with a surface, amino acids are transferred from the finger to the fingermark deposit. Amino acids once deposited and absorbed into a porous substrate become very stable within the paper due to their strong affinity to cellulose [190]. It is because of this stability that amino acids are one of the most desirable target molecules within latent fingermarks as they do not migrate significantly under dry conditions. This therefore generally allows for the development of clear fingermark detail when undertaking amino acid specific development [40].

Although amino acids are of great benefit on porous surfaces, they are often ignored when dealing with fingermark detection on nonporous surfaces due to the availability of reagents that target other components of the deposit [191]. However, recently it has been shown that amino acids can be of use for the detection of latent fingermarks on nonporous surfaces. Research has shown that an amino acid targeting technique could be advantageous over existing techniques when dealing with old or degraded latent marks (given the stability exhibited by the amino acids) [129].

3.1.2 RNA aptamers to amino acids

As discussed in Chapter 2, aptamers can be based on either DNA or RNA, with little overall difference observed in binding properties and characteristics. Initially, almost all aptamers were based on RNA as research progressed from ribozymes and the investigation of their catalytic properties and the continuous exploration of the RNA world theory [138, 139]. RNA

aptamers have since been investigated heavily due to the “seemingly greater variety of structures” adopted compared to DNA based aptamers [134].

The recognition of small molecules by RNA based aptamers is one area that has received numerous studies. This has mainly been in response to the substantial interactions shown between small molecules and RNA in many biological processes and also their increasing use as tools in molecular biology [192-194]. RNA aptamers have been successfully selected to a number of low molecular weight molecules including organic dyes, amino acids, various nucleotides and derivatives, and antibiotics [193].

Of these small molecules, amino acids have been consistently well researched for a number of reasons. First, many scientists have explored the potential of RNA aptamers to reflect amino acid-codon interactions in their sequence when being selected for binding to amino acids since the genetic code links individual amino acids with RNA sequences [193, 195]. Second, amino acids provide an ideal set of molecules for specificity testing of selected aptamers due to their minor structural differences. For example, aptamers selected to L-citrulline and L-arginine, which are similar in molecular structure, both bound their respective target with a K_d of 10 μM. However, when tested on the opposite amino acid, no affinity was detected [196]. Arginine has also been used to show the extremely high enantioselective properties of an arginine aptamer which bound to L-arginine 12,000 fold better than to its D-enantiomer counterpart [192]. The important roles in numerous biological systems undertaken by the interactions between amino acids and RNA have also fuelled research into amino acid binding aptamers. Better understanding of biologically relevant protein-RNA or RNA-amino acid interactions is thought to be possible by identifying and examining RNA sequences that bind specifically to amino acids [192].

The fact that there has been a large amount of detailed research into the selection of amino acid binding aptamers highlights amino acids as being very suitable and successful molecules for aptamers to target. Before the discovery of aptamers, RNA motifs showing specific affinity to amino acids had been demonstrated. Yarus [197] identified a binding site for arginine on the intron of the *Tetrahymena* self-splicing ribosomal precursor RNA. Specificity was highlighted by the preference of the L-enantiomer over its D- counterpart. Further, L-arginine has been found to bind specifically to the trans-activation response (TAR) element of the human immunodeficiency virus (HIV). L-Arginine bound TAR with a K_d of 4 mM with specificity

being underlined by the ability of L-arginine to block Tat peptide/TAR interactions while this was not possible with L-lysine and analogs of arginine [198].

The selection of RNA aptamers to free amino acids was first undertaken in 1993 – less than three years after the invention of aptamers and the SELEX protocol. The selection of aptamers to L-arginine was undertaken by Connel *et al.* [199] with the use of affinity chromatography. Binding sequences were found to exhibit K_d values of 0.2 – 0.4 mM for immobilised L-arginine and 1 mM for free L-arginine [199]. Arginine has been a staple for aptamer selection with a number of further selections carried out showing increased binding affinity, the ability to re-select aptamers to arginine and the further identification of arginine aptamers resembling TAR [192, 194, 200].

Although arginine has received the majority of aptamer investigations targeting amino acids, there are a number of other amino acids that have been used successfully for aptamer selection. L-valine aptamers were selected to investigate the specificity of aptamers to aliphatic amino acids. RNA aptamers bound L-valine with a K_d of 12 mM, which was shown to be at least 10-fold lower than when tested against analogous amino acids such as D-valine, L-leucine, L-alanine and L-threonine [201]. L-isoleucine binding aptamers were found to the have K_d values of 200-500 μM and were again highly specific, binding L-isoleucine up to 10 times better than other amino acids including valine and alanine [202]. The reselection of an original dopamine binding aptamer to a L-tyrosine binding aptamer was undertaken by Mannironi *et al.* [203]. In their selection, an RNA aptamer binding to L-tyrosine was derived with a K_d of 35 μM. Strong L-stereoselectivity was found while the L-tyrosine aptamer also bound L-tryptophan and L-dopa with similar affinity [203]. An RNA based aptamer to phenylalanine has been identified by Illangasekare and Yarus [204]. The strongest binding sequence was found to possess a K_d of 50 μM to the free amino acid phenylalanine with strong stereoselectivity illustrated by the non-specificity shown to the closely related amino acids tryptophan and tyrosine [204]. L-Histidine binding RNA aptamers have also been successfully selected. The average K_d of the selected sequences showed a binding affinity of 19 μM with between a 100 – 1000 fold increase in the dissociation constant to the L-configuration over the D-enantiomer [205].

3.1.3 Small molecule detection techniques using aptamers

Although extensive research has been undertaken to select aptamers to small molecules this has really only been to show the overall ability of aptamers to select against these molecules. Research into the actual application and further development of aptamers binding to small molecules has been limited. A possible reason for this as stated by McKeague and DeRosa [206] could be due to the fact that aptamers cannot bind to the smaller molecules with the high affinity needed for use in most biosensing techniques. While aptamers to small molecules generally bind with K_d values in the mid to low micromolar range, aptamers to larger molecules are able to bind with K_d values in the low nanomolar range [206].

Research that has further continued in the field of aptamer-based small molecule detection has, however, shown that high selectivity and sensitivity can be achieved using aptamers in various modified assay methods. The quick detection of cocaine and ATP in complex matrixes such as blood has been achieved with a modified sandwich assay. Aptamers selected to cocaine and ATP were cut in half at a point in the sequence which would not impede target binding. By splitting the aptamers, the two parts will equilibrate between its two dissociated parts and a folded associated complex. By exposing the two aptamer parts to the target the equilibrium will be driven towards the formation of the complex, therefore creating a "sandwich assay" from the two parts of the aptamer. With this technique the specific detection of cocaine and ATP was possible down to 1 μM [207]. Immobilised aptamers in sol-gel derived silica to theophylline and thiamine pyrophosphate have also highlighted a method for small molecule detection out of solution with the theophylline aptamer able to provide similar levels of affinity while immobilised as reported when used in solution [208].

3.1.4 The potential for amino acid detection using an aptamer-based reagent

The detection of amino acids by aptamers away from the common SELEX and specificity tests is an area that lacks research. This may be for the reasons stated earlier by McKeague and DeRosa [206], while the detection of targets of greater importance could well be overshadowing amino acid detection methodologies. Despite this lack of research, other aptamer detection based techniques that involve either the aptamer or target in a "dry state" have been trialled and should be briefly mentioned here. The successful use of DNA aptamer-

functionalised AuNPs in a simple "dipstick" test for the small molecules adenosine and cocaine has been identified by Liu *et al.* [188]. The aptamer-nanoparticle conjugates were left to dry on a layered membrane that also contained streptavidin to provide visualisation of a positive test (Figure 3-1). When placed into a spiked solution, the aptamer-nanoparticle conjugates are re-hydrated and when bound become separated and migrate to the streptavidin; when in a blank solution, the conjugates do not separate and cannot move to the streptavidin [188]. Limits of detection for this technique were in the region of 20 µM for adenosine and 10 µM for cocaine.

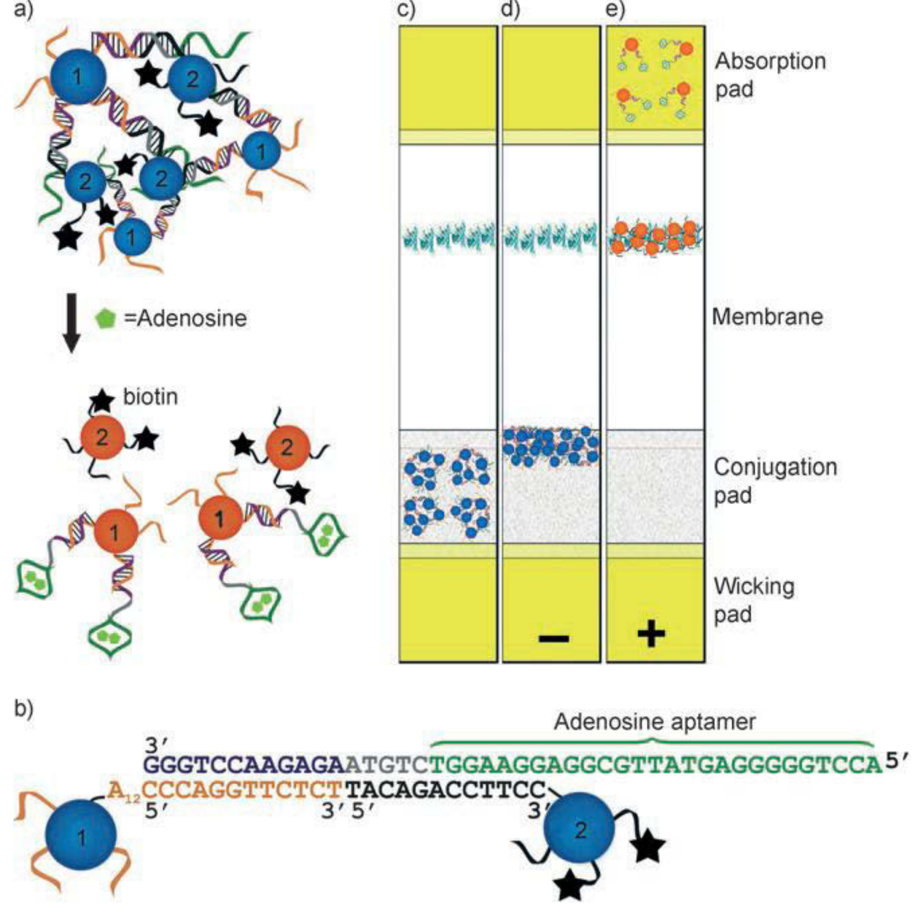


Figure 3-1: The aptamer-nanoparticle based lateral flow device used by Liu *et al*. Disassembly of nanoparticle aggregates to dispersed red-coloured nanoparticles when in the presence of adenosine (a). DNA sequence and linkages used for nanoparticle aggregates (b). Loaded lateral flow device with nanoparticle aggregates (shown on conjugation pad) and streptavidin (shown on membrane) before use (c), negative test (d) and positive test (e). Taken and reproduced from Liu *et al.* [188].

Balogh [209] selected aptamer sequences to the coat protein of the apple stem pitting virus (ASPV). Although not a small molecule (the ASPV molecular weight is 48 kDa), the methods used within the project clearly showed that aptamers can be used to detect dry targets

immobilised onto certain substrates. The ASPV was spotted onto nitrocellulose membrane and left to dry before being incubated in a protein-free blocking buffer. The substrate was then placed into a solution containing biotinylated aptamers at a concentration of 25 pmol/mL. Visualisation was achieved using ExtrAvidin-conjugated horse radish peroxidase solution and chemiluminescence [209]. Overall, this approach was able to detect dried ASPV spotted onto nitrocellulose membrane, limits of detection appeared to be around 100 ng of the native plant protein.

3.2 Objectives

The objective of the research detailed within this chapter was to investigate the feasibility of a fingermark reagent based on RNA aptamers that specifically targeted amino acids. This research includes the selection of published amino acid aptamer sequences that would be suitable for latent fingermark detection, while also identifying a method for providing visualisation of any subsequent development. In order for the reagent to be successful on commonly encountered fingermark substrates suitable methods for the application of the reagent were identified and tested. The identification of any non-specific interactions was also undertaken. The work in this chapter ultimately aimed to provide a "proof-of-concept" for the use of aptamers in latent fingermark detection and to determine the potential options for further research.

3.3 Experimental design

3.3.1 RNase

Ribonucleases, more commonly referred to as RNase, are necessary in living organisms for the removal of RNA no longer required by the cell; they are ubiquitous in living organisms and found to be extremely stable and robust [210]. There are many forms of RNase including RNase A, RNase H, and RNase P, all of which are found in different locations with highly specific, individual tasks. Some of these specific enzymatic activities include the metabolism of cellular RNA, anti-pathogen activities and as an ancestral host defence function. With these properties RNase can be used in biochemical procedures such as the isolation of

specific nucleic acids. With the addition of RNase into a nucleic acid preparation, RNA will be eliminated leaving behind the intact DNA [211].

RNase has recently been linked to playing a role in providing a chemical barrier for human skin to protect our bodies against the plethora of potentially pathogenic material encountered on a regular basis [212]. A number of antimicrobial peptides (AMPs) have been identified as important effector molecules in providing this chemical barrier [213]. Of these AMPs, at least two belong to the RNaseA100 superfamily, these being RNase 7 and psoriasin [214]. Psoriasin is a 11 kDa metal-binding protein that is secreted onto the surface of the skin through the sebaceous glands [215, 216]. The uppermost parts of the hair follicles and nose skin are areas where psoriasin is found at its highest concentration. Psoriasin is found to be most effective for antimicrobial activity against *Escherichia coli* but also shows activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* [216].

The second of the RNaseA100 superfamily is the 14.5 kDa antimicrobial ribonuclease RNase 7 [217]. RNase 7 is a highly basic cysteine-enriched protein that is constitutively expressed in normal stratum corneum [214]. It exhibits a broad spectrum of antimicrobial activity against both Gram-negative and Gram-positive strains at low micromolar concentrations including *S. aureus, P. aeruginosa, P. acnes* and *C. albicans* [217]. The detection of both of these ribonucleases on the surface of the skin and the fact that they are enzymatically active proteins supports the fact the human skin harbors ribonuclease activity [217].

With the ubiquitous nature of RNase and their inherent enzymatic features, extremely high levels of cleanliness and sterile working procedures are required when dealing with RNA sequences to prevent any degradation. To be able to work successfully with RNA, the use of certified RNase-free lab equipment is needed while the cleaning of surfaces requires the use of specific RNase decontamination solutions.

Due to the known presence of RNase activity on the human skin, it can be hypothesised that some RNase will be transferred when a latent fingermark is deposited. This therefore could be highly detrimental to any RNA based reagent used for latent fingermark detection. In order to assess the persistence of RNase within latent fingermarks, a specific RNase detection kit from Integrated DNA technologies, Inc. was used. This allowed for the investigation of

RNase activity within latent fingermarks, and also the persistence of possible RNase on porous and nonporous surfaces.

3.3.2 Selection of suitable sequences

As mentioned previously in Section 3.1.2, there has been a number of RNA sequences selected against various amino acids. The sequences all have different properties and have been found to bind their selected targets with different affinities and specificities. For a successful aptamer-based reagent, it was decided that two aptamer sequences should be selected with different properties. This would allow for comparisons to be made regarding what types of characteristics are needed by the aptamers for successful fingermark development.

Table 3-1 shows all the published RNA based aptamers selected to various amino acids with their length, binding affinity and specificity. As the table shows, the number of different amino acids targeted is actually quite small. Only eight amino acids have aptamers selected to them. Of these eight, there are only five that have been detected in latent fingermarks and these are all in low abundances, with histidine at a serine ratio of 17 the highest aptamer sequence available.

The L-histidine and L-isoleucine (2003 sequence) were the two aptamer sequences selected for reagent optimisation (Table 3-2). These two sequences were selected for a number of reasons. The L-histidine aptamer was chosen mainly because L-histidine is the most abundant amino acid for which an aptamer sequence is available. It also has a strong dissociation constant (12 µM) and is of medium length when dealing with aptamer sequences. In published studies, this aptamer also showed no affinity for any other targets, which potentially gives the reagent high selectivity in just targeting L-histidine.

Table 3-1: A list of all RNA aptamers selected to amino acids published in the literature to date (up until December 2012).

Target	Year	Length (mer)	Binding affinity (K_d)	Selectivity (K_d)	Reference
L-arginine	1993	72	0.2-0.4 mM	Guanosine 5′-monophospate (same K_d)	[199]
L-arginine	1994	44	56-76 μM	D-arginine (410 μM)	[194]
L-arginine	1996	113	330 nM	None reported	[192]
L-arginine	2010	17-50	0.5-4.4 mM	D-arginine (3-29 mM)	[218]
L-citrulline	1994	44	62-68 μM	D-citrulline (180 μM)	[194]
L-histidine	2005	40	12 μM	D-histidine (3-9.5 mM)	[205]
L-isoleucine	1998	50	200-500 μM	Norleucine (1 mM) L-valine (3.5 mM) L-alanine (3.5 mM) L-glycine (3 mM)	[202]
L-isoleucine	2003	22	2 mM	L-glycine (1.9 mM) Norleucine (3.5 mM)	[219]
L-isoleucine	2005	39	0.9 mM	Leucine and glycine (K_d not reported)	[220]
L-phenylalanine	2002	80	50 μM	L-tyrosine (32 μM) L-tryptophan (51 μM)	[204]
D-tryptophan	1992	120	18 μM	None reported	[221]
L-tyrosine	2000	93	23 μM	L-tryptophan (22 μM) L-DOPA (22 μM)	[203]
L-valine	1994	72	12 mM	L-alanine (210 mM) L-leucine (180 mM) L-isoleucine (230 mM) glycine (260 mM)	[201]

Although isoleucine only has a serine ratio of eight for amino acids in latent fingermarks, the aptamer sequence was selected mainly due to the affinity shown for glycine. Glycine is the second most abundant amino acid found within latent fingermarks, and although no sequence was found to this amino acid, the L-isoleucine aptamer does show affinity with a K_d

of 1.9 mM. The sequence was also chosen because of its short length and that it has almost a 1000 fold lower K_d compared to the L-histidine aptamer. Although it would be thought that sequences with stronger binding affinity would be most suitable, the fact that no data exists surrounding the use of aptamers in this approach meant that the binding affinity required for latent fingermark detection was unknown. The use of two sequences selected with such different binding affinities therefore allowed for an investigation into just what level of binding affinity is required for a successful aptamer-based latent fingermark reagent.

Table 3-2: RNA sequences for selected aptamers and predicted secondary structures.

Aptamer	K_d	Sequence	Predicted secondary structure
L-histidine [205] (Aptamer 1)	12 µM	GGCAUCGGAAAGUGGG UUGAUGUAAGUAACAG GCGAUGCC	
L-isoleucine [219] (Aptamer 2)	2 mM	ACUUCACAGGAGGAGCG UCGGU	

In order to detect binding of the aptamer to amino acids and fingermarks, a method of visualisation was required. As the aptamer sequences are chemically synthesised, the addition of fluorescent tags is an easy and very suitable method. Fluorescent tags as mentioned in Chapter 2 are also advantageous for use with aptamers as they do not interfere with the actual binding mechanisms of the aptamer/target complex. There are numerous fluorescent tags available and the choice of tag is ultimately dependent on what excitation and emission properties are required by the user. For this research, it was decided that a tag that had similar excitation and emission properties to current fingermark reagents would be most appropriate to allow for clear comparisons at a later stage in the research.

The fluorescent tag selected for this work was a hexachlorinated version of the fluorescent dye fluorescein called hexachloro-6-carboxyfluorescein (HEX). The dye is a phosphoramidite, which allows the dye to be easily attached to the 5′- end through a six-carbon chain linker once the desired sequence has been chemically synthesised [222]. This dye was chosen due to its absorbance maximum of 538 nm and emission maximum of 555 nm. As shown in Figure 3-2, the dye exhibits a secondary excitation peak at around 500-510 nm. It was felt that, with this secondary excitation band, the dye would be suitable for common forensic imaging systems.

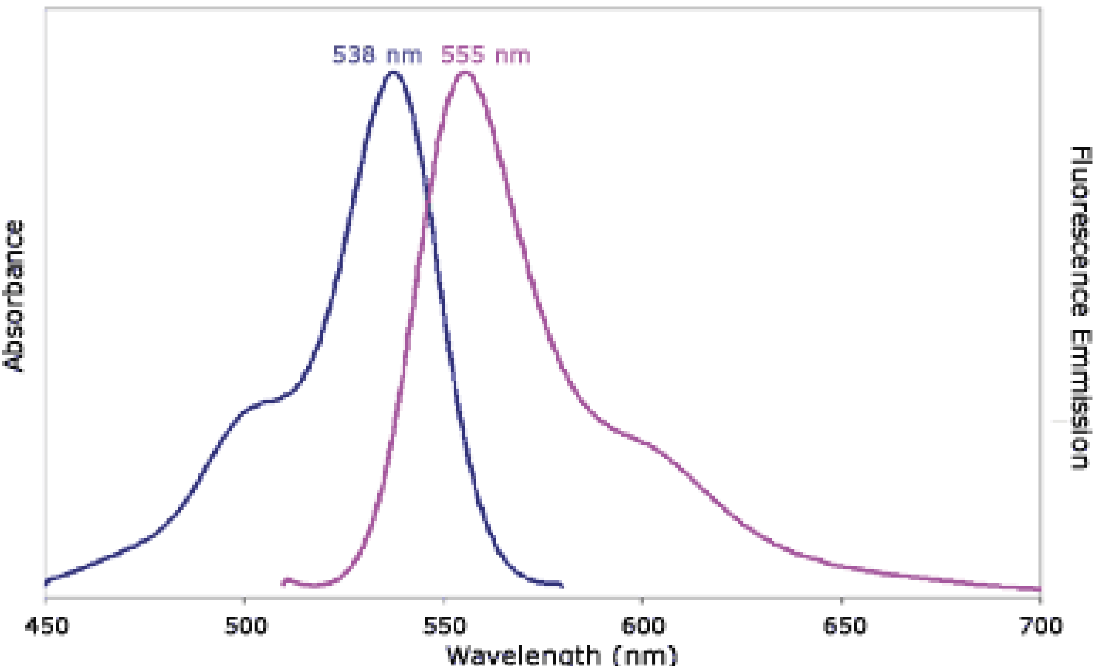


Figure 3-2: Spectra showing fluorescent absorbance and emission maxima for hexachloro-6-carboxyfluorescein. Taken and reproduced from Integrated DNA technologies Inc. [223].

With the sequences and dye selected, the aptamers were commercially synthesised by Invitrogen Life Technologies, USA. Both sequences were synthesised on a 200 nmole scale and were purified using HPLC. In order to be able to carry out several different experiments with the sequences, each aptamer was separated equally into four tubes. Aptamer 1 (L-histidine) contained approximately 148 µg of RNA material per tube while aptamer 2 (L-isoleucine) contained approximately 227 µg per tube. Both sequences were received lyophilised and were stored at -20 ^{O}C until required.

3.3.3 RNA dissolution and reagent optimisation

As it is well known RNA is easily dissolved in aqueous solutions due to its negatively charged phosphate backbone and the high polarity of water. The use of a largely aqueous solution for amino acid detection in fingermarks is impractical due to the high solubility of amino acids in water. Any attempt to use an aqueous reagent to target amino acids in a fingermark would result in the loss of detail as the amino acids will be removed from the surface. Most amino acid targeting fingermark reagents therefore rely on hydrofluoroethers (HFEs), or other common non-polar organic solvents, as the carrier solvent. The use of organic solvents for the dissolution of oligonucleotides, however, is extremely difficult due to the lower polarity of such solvents.

Early development of a method to solubilise the RNA in an organic solvent focussed on using mixtures with different solvent ratios to water. The use of a methanol-ethanol-buffer system showed that double stranded DNA was able to retain its duplex structure in a solution containing 50% methanol, 45% ethanol and 5% saline-sodium citrate buffer [224]. Although this solution may have dissolved the RNA oligonucleotides, it was hypothesised that the use of methanol and ethanol at such high concentrations may have a detrimental effect on the amino acids and cause migration of these compounds in a treated fingermark.

To investigate the potential of using organic solvents for dissolution, a study was undertaken comparing the ratio of water to organic solvent and its subsequent effect on amino acids placed on a porous surface. Amino acid standards were pipetted onto white copy paper, cellulose thin layer chromatography (TLC) plates and filter paper, and left to dry for one week at room temperature. The various solvent/water concentrations were then pipetted over each amino acid at 10 µL amounts. These were again left to dry for one day before being subjected to development with IND-Zn and visualisation under standard operating conditions (see Appendix II). The solvents tested and their relative ratios to water are shown in Table 3-3 together with the overall results.

Table 3-3: Results of solvent/H_2O ratio on IND-Zn developed amino acid standards (with a tick indicating no amino acid migration detected and a cross indicating amino acid migration).

		Solvent/H_2O ratios		
Solvent	**Surface tested**	70% solvent 30% H_2O	85% solvent 15% H_2O	95% solvent 5% H_2O
Methanol	White copy paper	×	×	×
	Cellulose TLC	×	×	×
	Filter paper	×	×	×
Ethanol	White copy paper	×	×	×
	Cellulose TLC	×	×	×
	Filter paper	×	×	×
Acetonitrile	White copy paper	×	×	×
	Cellulose TLC	×	×	×
	Filter paper	×	×	×
Acetone	White copy paper	×	×	✓
	Cellulose TLC	×	×	✓
	Filter paper	×	×	✓
DMSO	White copy paper	×	×	×
	Cellulose TLC	×	×	×
	Filter paper	×	×	×
Dioxane	White copy paper	×	×	×
	Cellulose TLC	×	×	×
	Filter paper	×	×	×

Overall, it was found that most of the solvents caused strong migration of amino acid standards even when the ratio of water was at 5% v/v. This is mostly likely due to the polarity of the solvents tested. A solution of 95% acetone and 5% H_2O was found to cause no diffusion of the amino acid spots and hence was selected for aptamer reagent dissolution trials.

Initial ssDNA oligonucleotide solvent dissolution tests were undertaken using ssDNA oligonucleotides. DNA was used due to the donation of some short oligonucleotides from the biology department that were surplus to requirements. The ssDNA oligos were received lyophilised and had not been used previously. Four oligos all of 22 mer and similar quantities (approx. 280 µg) were tested with the acetone/water solution. Two were tested by adding acetone/water (different ratios) directly into the tubes, followed by vigorous shaking, vortexing and heating. In the other tubes, water was added first with the acetone then being added after (resulting in different ratios). The results of these experiments are shown in Table 3-4.

Table 3-4: Results from initial dissolution trials with acetone/water solutions.

		Method used and result		
Sequence	**Solvent method**	**Shaking for 2 mins**	**Vortex for 2 mins**	**Heating @ 37 ^{O}C for 5 mins**
1	90% acetone 10% H_2O Total volume 100 µL	White pellet formed Clear solution	Pellet still visible Clear solution	Pellet still visible Clear solution
2	95% acetone 5% H_2O Total volume 100 µL	White pellet formed Clear solution	Pellet still visible Clear solution	Pellet still visible Clear solution
3	10 µL H_2O then slow addition of 90 µL acetone	Cloudy solution with pellet visible	Cloudy solution with pellet visible	Cloudy solution with pellet visible
4	5 µL H_2O then slow addition of 95 µL acetone	Cloudy solution with pellet visible	Cloudy solution with pellet visible	Cloudy solution with pellet visible

As can be seen from the results, the use of acetone and water was not able to dissolve the DNA. This may be due to acetone being an aprotic solvent and therefore being unable to donate hydrogen and allow full dissolution. It was found that a ratio of 85% H_2O and 15%

acetone was needed to allow for successful dissolution, which is obviously unusable for amino acid detection given the high content of water.

With the results obtained from the acetone tests, other polar protic solvents were trialled to see if any permitted full dissolution of the oligonucleotides. Although some had already been shown to cause migration of amino acids, it was decided to test them to see if any solvent/water combination would be suitable. Solvents tested were methanol, ethanol and isopropanol. These were tested in the same ratios of solvent to water as had been used in the acetone trials. Unfortunately, these solvents were also unable to dissolve the ssDNA oligos, with pellets clearly forming (Figure 3-3).

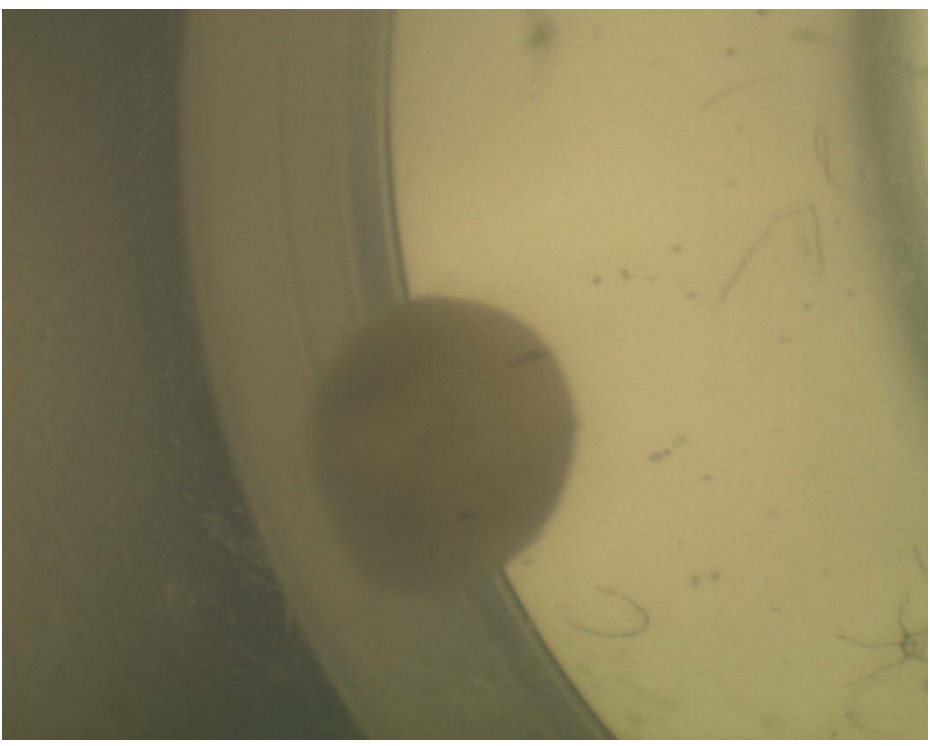


Figure 3-3: A DNA pellet formed after the trial to solubilise using 90% methanol / 10% H_2O. Image recorded using white transmittance light and viewed at x20.

As the solvent/water solutions were unable to dissolve the ssDNA oligonucleotides, another method was sought to permit the dissolution of oligonucleotides in organic solvent. The use of poly(ethylene glycol) (PEG) as a method to dissolve DNA in various solvents has been successfully used in a number of studies [226-228]. In two of the studies, DNA was obtained from salmon sperm before being incubated with different ratios of DNA/PEG (MW 3350). The solution was then freeze dried before using a variety of organic solvents including methanol,

DMSO, dichloromethane, chloroform and DMF to successfully dissolve the DNA/PEG nanocomplexes [226, 228]. In the other study, a chemically synthesised ssDNA oligo was modified through the incubation with PEG-NHS ester for several hours before being purified by reverse-phase HPLC; dissolution was achievable with 1,2-dichloroethane [227].

As PEG is a hydrophilic and non-ionic polymer, it has been found to interact with proteins/DNA by hydrogen bonding and/or hydrophobic interactions when used at low concentrations (< 8% w/v) [229]. Through these interactions, proteins are stabilised in aqueous solutions due to preferential hydration of the proteins [228]. This therefore allows PEG to form nanocomplexes with proteins/DNA which are then soluble in organic solvent due to PEG's solubility in both water and organic solvents [230]. Once solubilised, the tertiary DNA structure has been found to be reserved and stable [227, 228].

The PEG approach used by Mok *et al.* [228] was selected as the most suitable method as it required no post HPLC clean-up of the DNA. Although Mok *et al.* found that PEG of a molecular weight of 3350 and at a ratio of 1:15 (DNA:PEG) was most successful in allowing full dissolution, it was decided to test some variations for this project due to the differences in oligonucleotides. Whereas Mok *et al.* used ssDNA obtained from salmon sperm, which is approximately 2000 bases long, the DNA and RNA to be used for this project were far shorter (< 50 bases).

Two different PEGs were chosen for initial DNA trials, these being PEG MW 3350 and PEG distearate MW 400. Different DNA:PEG ratios were also investigated, with ratios of 1:15, 1:20, 1:25 and 1:30 tested. In order to test the ratios, both PEGs were dissolved into stock standards of 1 µg/µL to allow for precise addition to the already solubilised DNA. Once all DNA samples had been mixed with the selected PEG, they were left to incubate at room temperature for four hours before being frozen overnight. All samples were then lyophilised at −50 ^{O}C under a pressure of 1 X 10^{-1} Torr. Methanol was then used for dissolution as this solvent had been shown to dissolve ssDNA very well [228].

Overall it was found that PEG MW 3350 was more suitable for the dissolution of DNA and at a ratio of 1:20 or higher. In testing, it was seen that the PEG distearate 400 was unable to allow full dissolution of the nanocomplexes, with small strands still visible when viewed under a microscope. This was further demonstrated when the samples were tested for purity and

quantification using a spectrophotometer. Results showed a much higher absorbance reading compared to the initial reading before PEG was added, highlighting that the DNA is not fully dissolved (Figure 3-4).

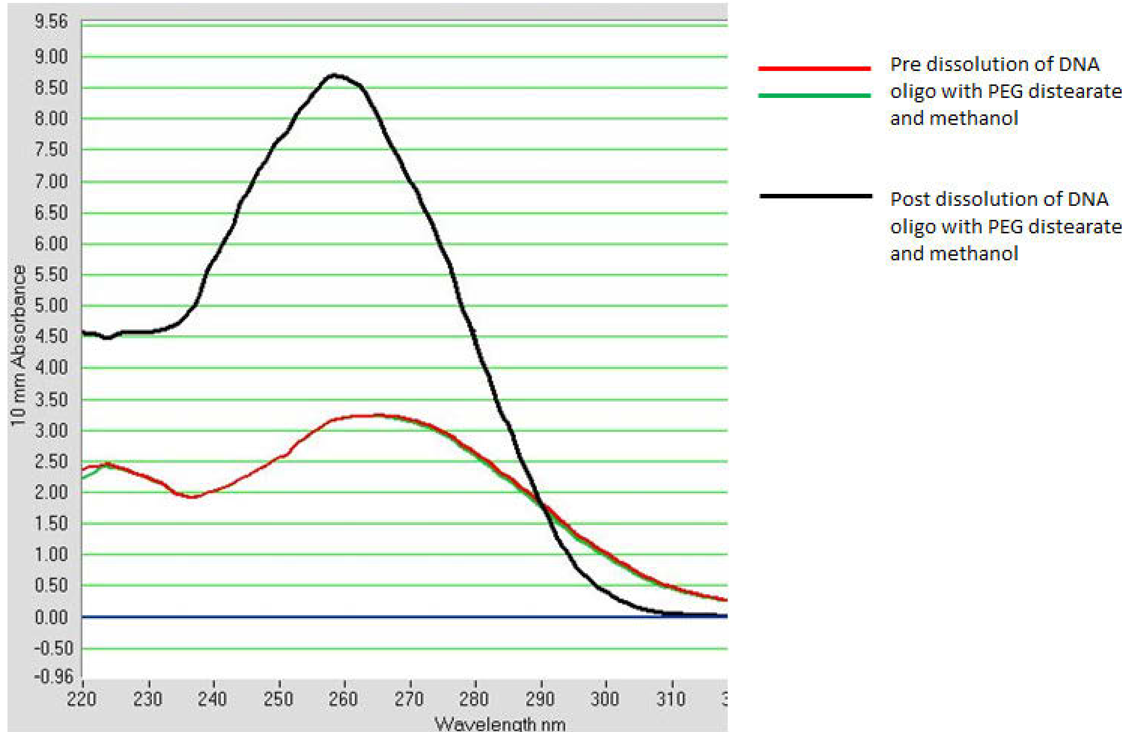


Figure 3-4: Spectrophotometer result of ssDNA (30 mer) both pre and post dissolution with PEG distearate MW 400 and methanol.

The use of PEG MW 3350 provided much better results with full dissolution of the DNA/PEG nanocomplexes as shown in Figure 3-5. A ratio of 1:20 DNA to PEG w/w was also shown to be needed. Full dissolution was not possible with any ratio below this.

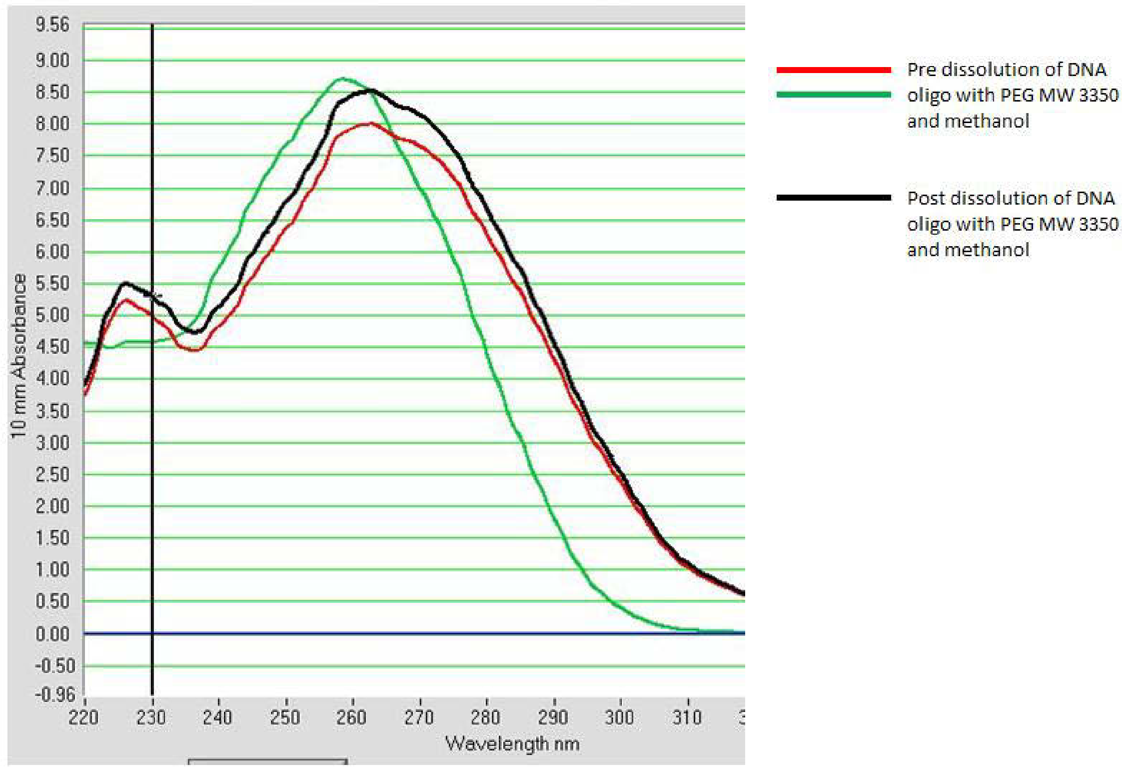


Figure 3-5: Spectrophotometer result of ssDNA (30 mer) both pre and post dissolution with PEG MW 3350 and methanol.

It was therefore decided that the most suitable method for dissolution would be to use PEG MW 3350 and at a ratio of 1:20 using methanol as the solvent. This method also proved to be relatively stable, with the material remaining completely solubilised with similar spectra being obtained after four weeks of the oligos being in solution. A full investigation of the oligo structure, however, could not be undertaken due to the required equipment not being available.

The PEG technique was then trialled on the RNA aptamer sequences using exactly the same method. The aptamer sequences were also diluted with methanol to create a stock solution of 100 µL. For both sequences, the PEG technique was able to allow full dissolution of the RNA sequences as shown in Figure 3-6 and Figure 3-7. When the aptamer solutions were not being used, they were kept in a dark container and stored at 4 ^{O}C.

In order to help protect and stabilise the aptamer sequences, metal salts were added to the stock solution. 0.1 mM Zn^{2+} and 7.5 mM Mg^{2+} diluted in methanol were included in the stock solution. The selection and concentration of these divalent cations was obtained from the

literature, which strongly suggested that both of these metal salts help to stabilise the RNA structure [231] and help improve the binding affinity of the aptamers [202].

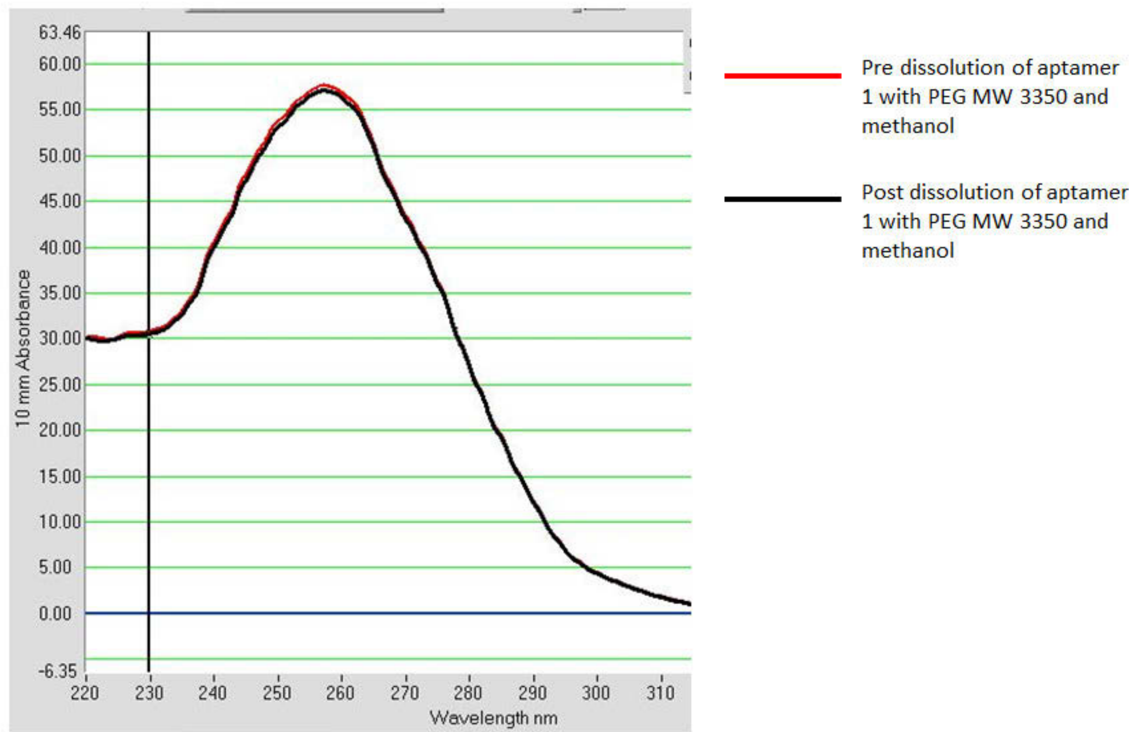


Figure 3-6: Spectrophotometer result of aptamer 1 both pre and post dissolution.

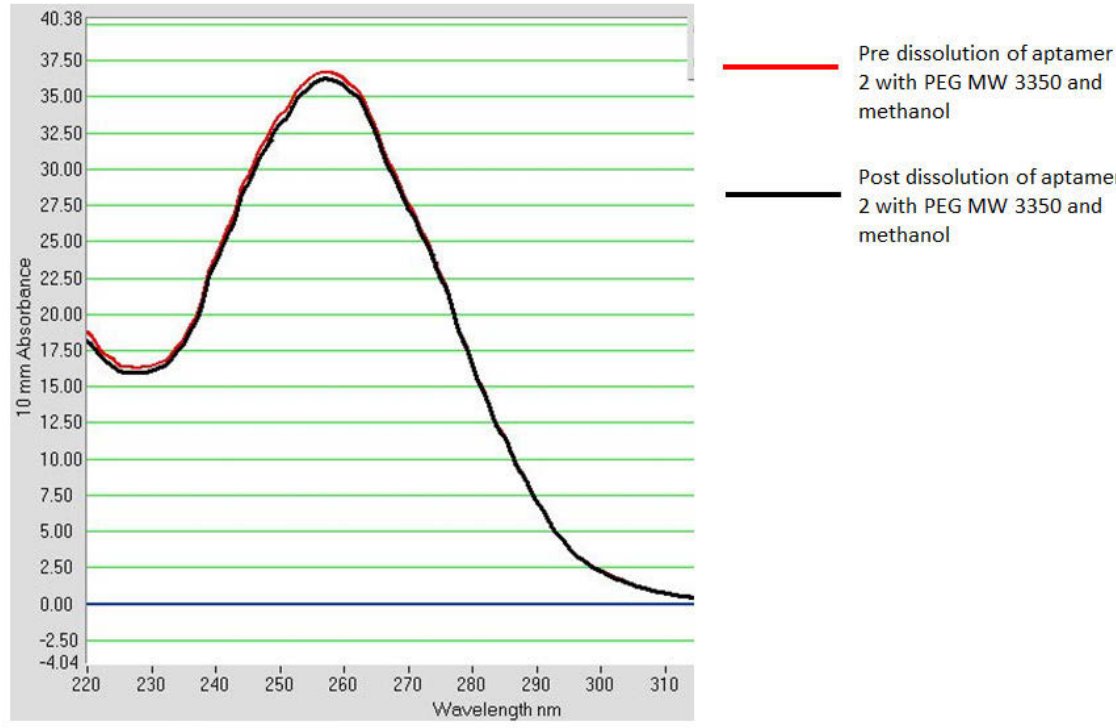


Figure 3-7: Spectrophotometer result of aptamer 2 both pre and post dissolution.

3.3.4 Identification of appropriate surfaces

Initial use of the aptamer-based reagents focussed on the detection of amino acids on porous surfaces including standard virgin white copier paper, cellulose TLC plates and laboratory standard cellulose filter paper. Surfaces were pipetted with 2 µL of amino acid solution of various concentrations ($x10^{-1}$ – $x10^{-6}$ M) and left to dry for at least 24 hours. After, the substrates were tested by adding 2 µL of aptamer reagent over each amino acid deposit followed by visualisation (Figure 3-8). When visualised, it was clear that the aptamer reagent showed strong affinity towards the cellulose substrate. Many washing methods with a range of solvents (methanol, ethanol, acetone, acetonitrile and water) were tested but the excess reagent was unable to be removed. The cause of this is most likely due to hydrogen bonding interactions occurring between the phosphate backbone of the RNA and the cellulose substrate.

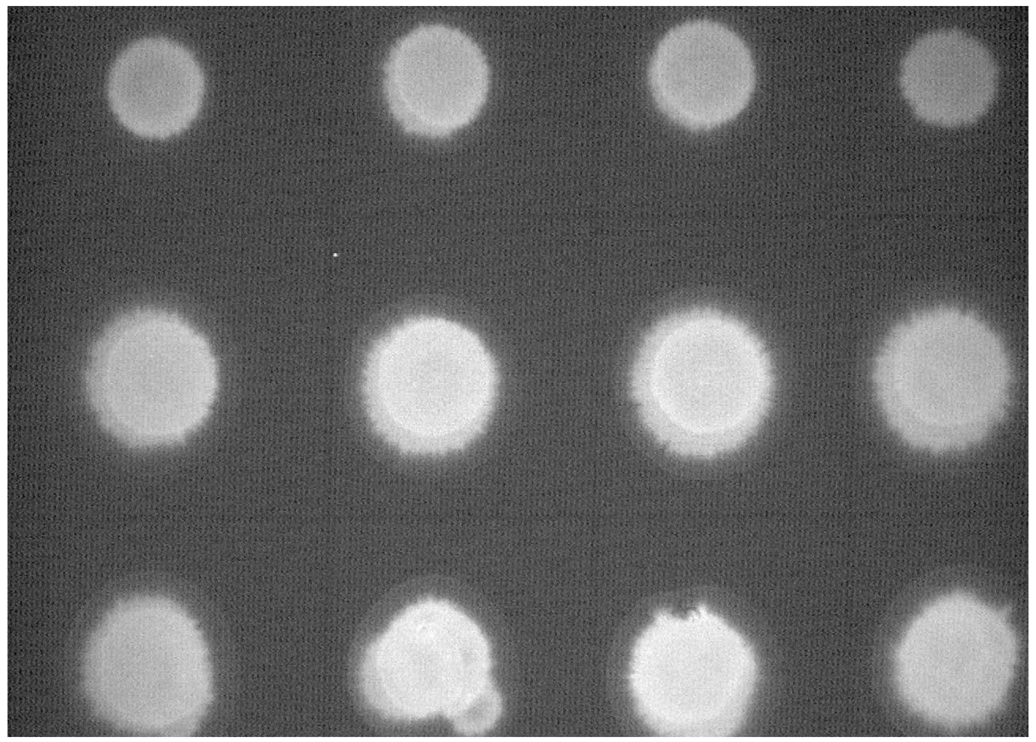


Figure 3-8: Amino acid spots on TLC plate developed using aptamer 2 at a concentration of 0.05 mM. Amino acids (L-R): Ile, Val, His, Ser. (top-bottom): 0.1, 0.01, 0.001 M. Image recorded in the fluorescence mode using a 485-535 nm band-pass filter for excitation with a 550 nm long-pass camera filter.

Because of this interaction, it was decided that a more suitable test surface that was nonporous and inert would be needed. The use of glass slides for the successful detection of amino acids using antibodies has already been shown [129]; therefore, glass slides were selected for the continuation of this study. As has already been highlighted (see Section 3.3.1), RNase is ubiquitous and, to ensure that RNase activity was inhibited, all glass slides employed in this study had to be meticulously clean.

Pathology grade glass slides were cleaned first by sonication for 10 minutes in acetone before being immersed for five minutes in hospital grade antimicrobial cleaning solution. Slides were then placed into an oven set at 100 ^{O}C to dry thoroughly for two hours. All slides were then stored in a specific slide holder that had been cleaned using the antimicrobial cleaning solution.

3.3.5 Collection of fingermarks

Fingermark samples were collected from a small group of 10 donors, five female and five male. Donors were asked to place both natural and charged marks on single glass slides and also across two slides (split impressions) to allow for selectivity testing. Natural fingermarks were obtained from donors who had not washed their hands for approximately two hours and had generally refrained from touching their face and hair excessively in that time. Charged marks were obtained from donors by asking donors to ‘charge’ their fingers by running them across the forehead, nose or back of the neck before placing the marks. Donors were asked to place their ring, middle and fore finger on the glass slides with gentle pressure as to not cause any obliteration of the fingermark ridges.

Fingermark samples were also taken on standard white copier paper to allow for testing with existing amino acid targeting reagents to give an understanding of the “type” of fingermarks the donors deposited. It was generally seen that the male donors produced fingermarks that were of greater and more continuous detail than those achieved from the female donors; however, this was not always the case, with different results being obtained from the same donor on a regular basis. This reiterated the fact that large natural variances in fingermark constituents from the same donor are very common and this factor needs to be taken into consideration [26, 232, 233].

As the sole intended target for the aptamer-based reagent were amino acids, eccrine groomed marks were also collected from one female and two male donors. The method for the collection of groomed marks was followed from standard published methods [234]. Donors were asked to wash their hands thoroughly with soap and excess water to ensure no soap residues were remaining on the hands and fingers. Once dried, donors’ hands were placed into clean polyethylene bags and sealed for 30 mins. Marks were then deposited in the same manner as previously discussed. All fingermarks were stored in a clean microscope slide holder.

3.4 Materials and methods

3.4.1 General

3.4.1.1 Reagents

RNA aptamers selected to L-isoleucine and L-histidine were synthesised by Invitrogen Life Technologies Inc., USA. Fluorescent tag modifications were undertaken during the synthesis. Aptamers were received lyophilised and used as supplied.

L-Serine [CAS 56-45-1]; L-histidine [CAS 71-00-1]; glycine [CAS 56-40-6] (ReagentPlus®, ≥99% (TLC)); L-isoleucine [CAS 73-32-5]; L-leucine [CAS 61-90-5]; L-phenylalanine [CAS 63-91-2]; L-theronine [CAS 72-19-5]; L-valine [CAS 72-18-4] (reagent grade, ≥98% (TLC)); poly(ethylene glycol) [CAS 25322-68-3], BioUltra, MW 3,350; poly(ethylene glycol-400) distearate [CAS 9005-08-7]; magnesium chloride [CAS 7786-30-3] (anhydrous, ≥98%); zinc chloride [CAS 7646-85-7]; 5(6)-Carboxyfluorescein BioReagent (≥95% HPLC); squalene [CAS 111-02-4] (≥98%, liquid); oleic acid (≥99% GC) and palmitic acid (≥99%) were obtained from Sigma-Aldrich and used as supplied.

D-Glucose [CAS 921-60-8] (anhydrous, UNIVAR) was obtained from Ajax chemicals and used as supplied.

MO BIO laboratories Inc. Molecular Biology Grade Water was obtained from GeneWorks and used as supplied.

1,2-Indanedione-zinc working solution was prepared and employed as stated in the NCFS workshop manual [225] (see Appendix II).

22 mer ssDNA oligonucleotides synthesised by Invitrogen Life Technologies Inc., USA, were received lyophilised and used as supplied.

KERR CaviCide® hospital grade disinfectant/decontaminant cleaner was used as supplied.

RNaseAlert™ Kit was obtained from Integrated DNA Technologies Inc., USA, and used as supplied.

Acetone [CAS 67-64-1] and isopropanol [CAS 67-63-0] were obtained from Chem-Supply and used as supplied.

Methanol [CAS 67-56-1] and absolute ethanol [CAS 64-17-5] were obtained from AnalaR NORMAPUR® VWF and used as supplied.

Acetonitrile [CAS 75-05-8] HPLC grade was obtained from Scharlau and used as supplied.

3.4.1.2 Instrumental

MO BIO laboratories Inc. UltraClean™ certified RNase-free gloves were obtained from GeneWorks and used throughout the study.

A Julabo ShakeTemp SW22 waterbath was used for the incubation of substrate tubes during RNase testing.

A Rofin Poliview IV imaging system with a Rofin PL500 Polilight forensic light source was used for the visualisation of treated amino acid spot tests and fingermarks.

A Foster + Freeman video spectral comparator 6000 was used for the visualisation of treated amino acid spot tests.

Sarstedt 25 cm 2-position blade cell scrapers were used for the collection of latent fingermark deposit on glass slides.

A Singer magic steam press 7 was used for the development of samples treated with IND-Zn.

A Branson 2210 sonicator was used in oligo dissolving experiments.

A Ratex instruments vortex mixer was used in oligo dissolving experiments.

A Leica EZ4D microscope was used for the inspection of oligos in organic solvents.

A Memmert laboratory oven was used for the drying of microscope glass slides and amino acid standards at various temperatures.

A Kelvinator opal fridge/freezer was used for the storage and freezing of RNA samples.

A Thermo scientific NanoDrop 3300 full-spectrum fluorospectrometer was used for the analysis of DNA and RNA samples.

A Dynavac freeze drying unit model FD1 was used for the freeze drying of DNA and RNA samples.

Greiner bio-one microplate 96 well flat bottom ELISA plates were used for ELISA experiments.

A Budget airbrush-0.5 mm, double action, suction fed airbrush connected to a BOC scientific air regulator was used for the application of aptamer reagents.

A Paton scientific encounter orbital shaker was used for the mixing of RNA samples.

A glass thin-layer chromatography jar was used for the washing of developed glass slides.

Colourfrost plain edge glass microscope slides were obtained from HD scientific supplies Ltd.

Cellulose fibre TLC plates (0.1 mm) on PET foil (20 x 20 cm) were obtained from Fluka analytical.

Ultra white 80gsm copier paper was obtained from Reflex, Australia.

Whatman #1 ashless filter paper were obtained from Whatman.

3.4.2 RNase

Glass microscope slides were first cleaned by sonication in acetone for 10 minutes before being immersed in hospital grade antimicrobial cleaning solution for five minutes. The slides were then heated in an oven for two hours. White copier paper was selected from the centre section of a new packet and always handled with RNase free gloves. Five donors (two male

and three female) were asked to place both fresh (no prior loading) and charged (fingers run across the forehead) fingermarks onto each substrate with normal pressure and for a contact time of approximately two seconds. Samples that were to be tested as fresh were analysed the same day. Those that were aged were placed into a clean container and stored in the dark at room temperature for three weeks. Control samples for both surfaces were created by adding 1 µL of RNase A onto the surface and left to dry naturally.

Fingermarks placed on paper were analysed by first being cut into small sections and placed into 1.8 mL CyroPure plastic vials containing 1.5 mL RNase free water. These were then incubated for one hour at 37 ^{O}C. The RNase control surface was subjected to the same protocol. Using the RNaseAlert kit, 45 µL of sample from the vials containing the paper was added to the supplied substrate tubes. 5 µL of 10x RNaseAlert buffer was then added to the substrate tube and shaken. The substrate tubes were then incubated at 37 ^{O}C for one hour.

To obtain the contents of the fingermarks placed onto the glass slides, the samples were first wetted with approximately 30 µL RNase free water. A cell scraper was then used to run across the glass surface creating a pool at one end of the slide which was transferred to a 500 µL microcentrifuge vial. The process was repeated until over 50 µL of sample was achieved. The RNase control was subjected to the same protocol. The preparation of the samples was undertaken as shown above for the paper samples.

Positive and negative control samples were also created using the kit. 5 µL of 10x RNaseAlert buffer was added to both substrate tubes before the inclusion of 45 µL of RNase free water. For the positive control, 1 µL of RNase A was added. Visualisation of all the results was undertaken using a Rofin Poliview system with an excitation wavelength of 350 nm. Results were recorded both with and without a camera filter. The camera filter used was a 555 nm bandpass filter.

3.4.3 Development of aptamer-based reagent

Both sequences were first re-hydrated with 100 µL of RNase free water, shaken for one minute then spun down in a centrifuge for 30 seconds. Estimated RNA quantity in each tube was obtained using a spectrophotometer with 2 µL of oligo solution being run for analysis. PEG (MW 3350) was dissolved in RNase free water at a concentration of 10 µg/µL. This PEG

solution was added to each sequence at a ratio of 1:20 (RNA/PEG) w/w. The RNA/PEG solution was left at room temperature for one hour with continuous agitation to create a clear liquid. After one hour, the tubes were placed into a freezer until the contents were completely frozen.

Once frozen, the tubes were freeze-dried at -50 ^{O}C under a pressure of 1 X 10^{-1} Torr. At all times the tubes containing the aptamer sequences were kept out of contact with light by using aluminium foil. After 12 hours the RNA/PEG solution was completely lyophilised and removed from the freeze-dryer. 0.5 mM stock solutions for both aptamer sequences were created with the addition of methanol. 0.1 mM Zn^{2+} and 7.5 mM Mg^{2+} diluted in methanol was further added to the stock solution. Working solutions were obtained by the dilution of the stock solutions with extra methanol, these solutions being of 0.1 mM and 0.05 mM and 2.5 µM concentrations. All solutions were stored at 4 ^{O}C when not being used.

3.4.4 Application of reagent to amino acids and fingermarks

Amino acid standards were made using RNase free water. Amino acids tested were L-histidine, L-valine, L-phenylalanine, L-serine, L-isoleucine, L-leucine, L-alanine, L-lysine, L-theronine and glycine. All of the standards were made up to a 0.1 M concentration, 10-fold dilutions were then conducted for all of the standards to create standards of 0.01 and 0.001 M. 2 µL of each amino acid solution was spotted onto the glass slide which had been pre-cleaned as detailed in Section 3.3.4. The slides containing the amino acids were then placed in an oven set at 40 ^{O}C for 1.5 hours. Fingermarks were collected as detailed in Section 3.3.5.

The aptamer solutions were applied to the amino acids and latent fingermarks in one of two ways. Initially the solution was applied using a pipette. 4 µL of the solution at 0.05 mM was applied over the dried amino acid spot and left to dry. The pipette method was also used on the latent fingermarks with 20 µL of the 2.5 µM solution being applied over the entire fingermark. Visualisation in the fluorescence mode was then undertaken with use of the Poliview with a 505 nm excitation source and 555 nm camera filter.

Application of the aptamer solution at 2.5 µM was performed with the use of a small adapted airbrush (Figure 3-9). Using a pipette tip as a reservoir for the reagent, 60 µL of reagent was added and propelled through the airbrush using compressed air. In a sweeping motion, the

solution was applied over the entire latent fingermark. The aptamer solution was again left to dry before being visualised as detailed above.

Figure 3-9: Adapted airbrush used to apply aptamer reagent to latent fingermarks on glass slides.

A wash step was undertaken after initial development using a solution of acetone and methanol in a 90:10 v/v ratio. This was applied, after the aptamer solution had dried, either by use of a pipette or by dipping. 150 µL of the solution was run over the top of the developed slides and allowed to run off. This process was generally repeated three times with images taken after every wash. The dip method involved placing the slide fully into the acetone:methanol solution for 5 seconds. Again this was generally repeated three times with images taken after every wash.

3.4.5 Investigating non-specific binding

Non-specific binding was investigated in two ways; the non-specific binding of the aptamer to other fingermark targets was undertaken using a modified ELISA, while the potential binding activities of the attached HEX dye was also investigated.

The wells of an ELISA plate were individually loaded with 200 µL of a specific target. The targets tested were L-histidine, L-isoleucine, L-serine, L-phenylalanine, glycine and glucose, all

of which were diluted to a concentration of 0.1 M in water. Also tested were squalene, palmitic acid and oleic acid which were diluted in ethanol to the same concentration. Wells containing no target were used as blanks. Once loaded, the plate was tightly sealed with plastic wrap and stored at 4 ^{O}C overnight. Wells were washed with 200 µL of the 90:10 acetone:methanol wash solution which was tipped out after the wash. This wash step was repeated up to three times before allowing the wells to dry. 20 µL of the aptamer solution was added to each well, with both aptamer solutions being used separately. The aptamer solutions were left to incubate for three hours while being continuously agitated. After the incubation period, the wells were washed again using the acetone:methanol wash. Results were recorded using the Polilight with an excitation wavelength of 505 nm and a 555 nm band pass camera filter.

5(6)-Carboxyfluorescein (FAM) was obtained from Sigma-Aldrich and dissolved in methanol to the same concentrations as the aptamer solutions. PEG was also added to the dye solution in the same quantity as had been used in the initial dilution of aptamer 2. The dye solution was applied to both amino acids and latent fingermarks using the same methods as previously stated in Section 3.4.4. The dye solution was also applied to split marks which were obtained by donors applying a fingermark across 2 glass slides placed together. Wash procedures were followed for dye stained marks as has been stated in Section 3.4.4.

3.5 Results and discussion

3.5.1 RNase

Two surface types were tested for the persistence of RNase, these being virgin fibre white copier paper (Reflex brand) and clear pathology grade glass microscope slides. The RNase Alert detection kit (Integrated DNA Technologies, Inc.) is a simple to use kit designed to give easy to read results when excited with a 350 nm light source. The kit contains a reporter molecule and a quencher molecule attached to either ends of an RNase specific oligonucleotide. On contact with RNase, the reporter molecule is cleaved producing a visible yellow/green emission once illuminated (Figure 3-10) [235]. The kit can also be used as an assay to give qualitative and quantitative data but this was not undertaken in this study due to the results obtained.

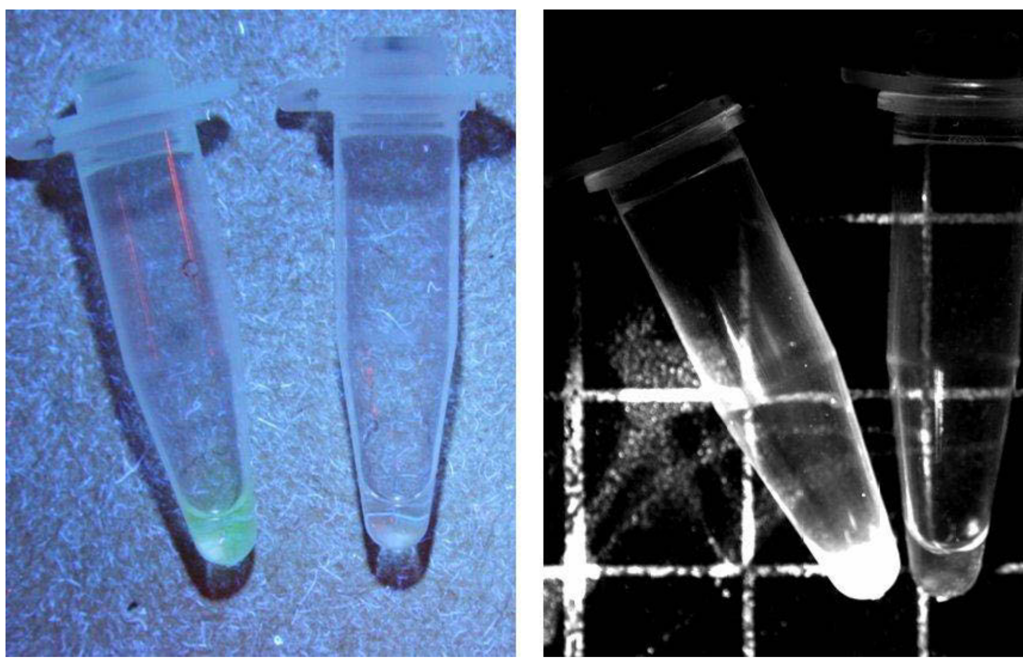


Figure 3-10: RNase positive (left) and negative (right) controls. Image recorded in the fluorescence mode using a 350 nm excitation light with a 555 nm band-pass camera filter.

Results from the paper surface for both fresh and aged marks were all negative, with no fluorescence visible in any sample as shown in Table 3-5. Negative results were also seen for both charged and natural marks. The test paper samples containing the RNase control did fluoresce brightly, illustrating that the protocol used for the extraction of any RNase from the paper surface was effective (Figure 3-11).

Table 3-5: RNase results for donor fingermarks on white copier paper (with a tick indicating a positive result and a cross indicating a negative result).

	Fingermark type and age			
	Fresh		Aged (3 weeks)	
Donor	Natural mark	Charged mark	Natural mark	Charged mark
1	✘	✘	✘	✘
2	✘	✘	✘	✘
3	✘	✘	✘	✘
4	✘	✘	✘	✘
5	✘	✘	✘	✘
RNase test	✓		✓	

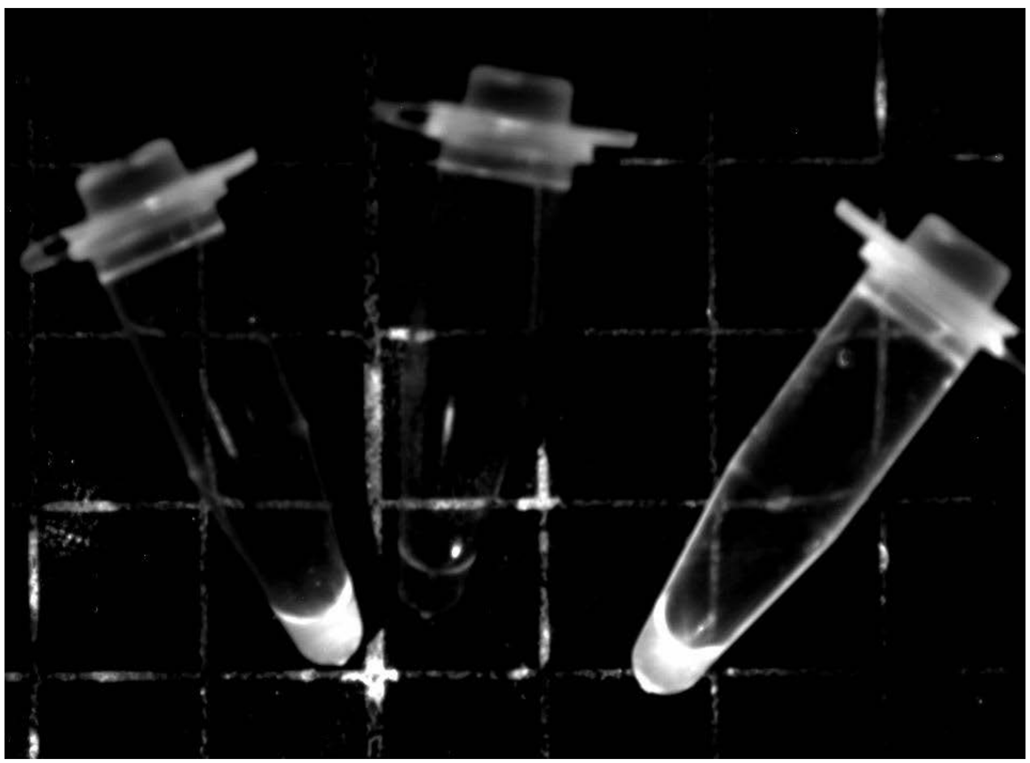


Figure 3-11: RNase positive control (left), negative (middle), RNase test from paper (right). Image recorded in the fluorescence mode using a 350 nm excitation light with a 555 nm band-pass camera filter.

Results from the glass surface also provided negative results from both fresh and aged donor fingermarks. Natural and charged fingermarks were all negative as well (Table 3-6). Again, test samples with the RNase control placed on the surfaces fluoresced strongly when collected, showing that the protocol for extracting RNase from the glass surface worked correctly (Figure 3-12).

Table 3-6: RNase results for donor fingermarks on glass microscope slides.

	Fingermark type and age			
	Fresh		Aged	
Donor	Natural mark	Charged mark	Natural mark	Charged mark
1	✘	✘	✘	✘
2	✘	✘	✘	✘
3	✘	✘	✘	✘
4	✘	✘	✘	✘
5	✘	✘	✘	✘
RNase test	✓		✓	

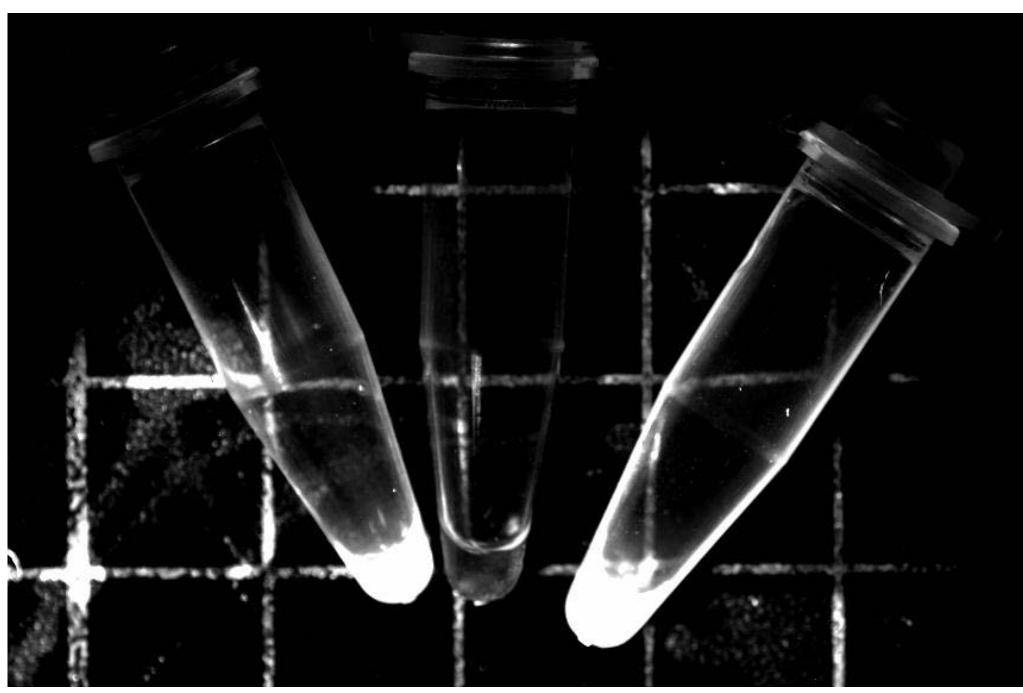


Figure 3-12: RNase positive control (left), negative (middle), RNase test from glass (right). Image recorded in the fluorescence mode using a 350 nm excitation light with a 555 nm band-pass camera filter.

The results obtained from the RNase tests all showed that there was no evidence of any RNase activity on either the latent fingermarks or the surfaces used. Although RNase has been well documented as being a ubiquitous and persistent enzyme, it did not appear to be present on the test samples in this study. With these results, it is safe to say that the RNA aptamers being used for latent fingermark detection will not be degraded or affected due to RNase activity.

3.5.2 Performance of the aptamer-based reagent on amino acids and fingermarks

As mentioned previously (see Section 3.3.4), the use of porous surfaces was not feasible in the initial testing of the aptamer reagent due to the background interaction of the aptamer and the cellulose substrate. Results from the porous surfaces did, however, provide details into what level of aptamer concentration was required to achieve suitable visualisation. It was observed that, at a concentration of either 0.5 or 0.1 mM, the fluorescence obtained from the aptamer solution was very strong at low imaging integration times (Figure 3-13). With this, it was decided that the initial tests undertaken with the aptamer solution should start with a concentration of 0.05 mM.

Initial results on glass slides were quite promising as some interaction between the aptamer reagents and the amino acids was seen, with some amino acids becoming fluorescent after aptamer development. The reagents appeared to show differing affinity to the amino acids. Aptamer 1 (which in published research was shown to only have affinity for L- histidine) produced visible fluorescence with histidine, phenylalanine, lysine and leucine at amino acid concentrations of 0.1 M (Figure 3-14). Amino acids of lower concentrations were not visualised with the reagent.

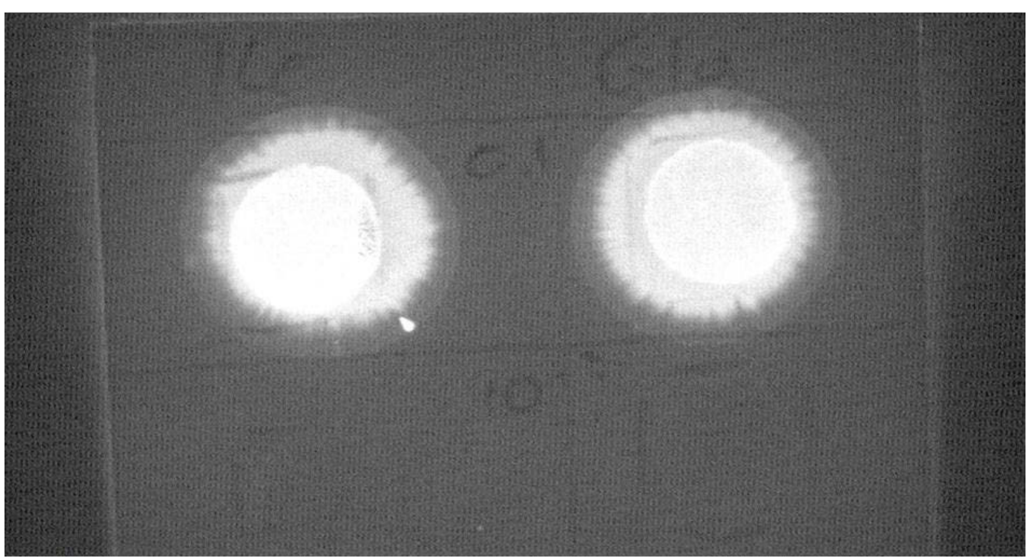


Figure 3-13: Isoleucine and glycine at 0.1 M visualised with aptamer 1 at 0.5 mM. Image recorded in the fluorescence mode using a 485-535 nm band-pass filter for excitation with a 550 nm long-pass camera filter.

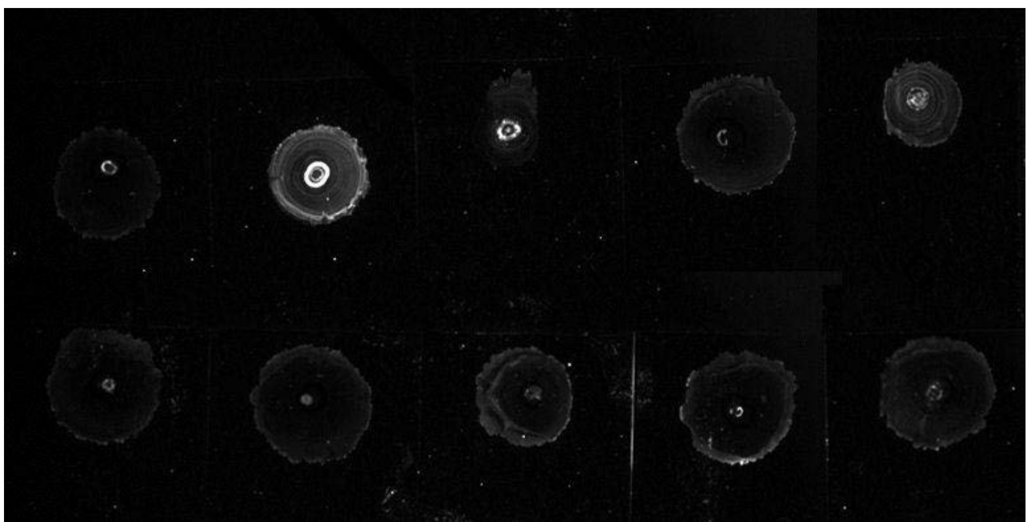


Figure 3-14: Amino acid standards (0.1 M) on glass slides developed with aptamer 1 at 0.05 mM concentration. Clockwise from top left: His, Phe, Lys, Gly, Leu, Ile, Ala, Val, Ser, Thr. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

The aptamer 2 reagent (which in published research showed affinity to L-isoleucine and glycine only) gave visible fluorescence with leucine, histidine, serine, glycine, isoleucine and phenylalanine. This reagent, however, was able to provide fluorescence with amino acid standards at both 0.1 and 0.01 M (Figure 3-15 and Figure 3-16).

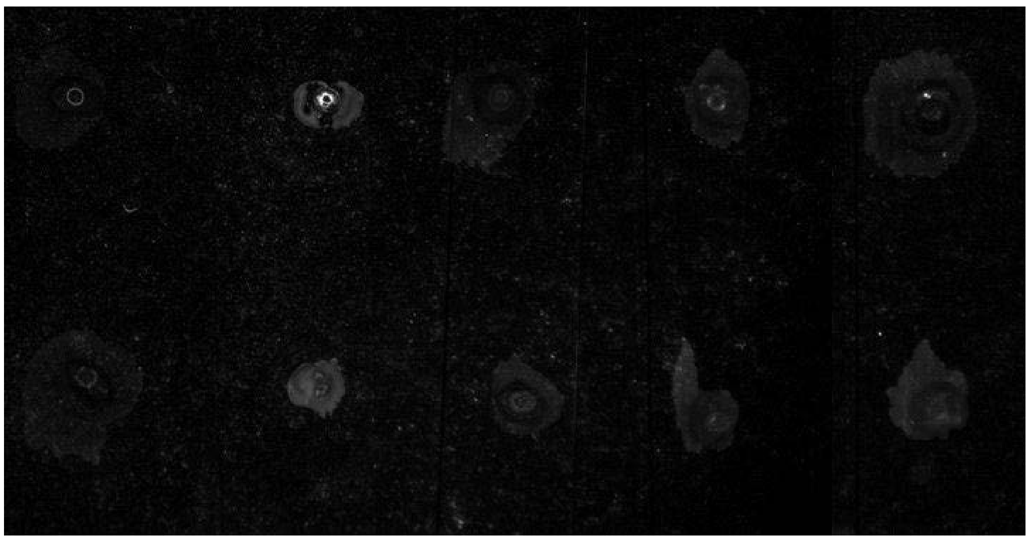


Figure 3-15: Amino acid standards (0.1 M, top, 0.01 M bottom) on glass slides developed with aptamer 2 at 0.05 mM concentration. From left to right: Ala, Leu, Lys, Thr, Val. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

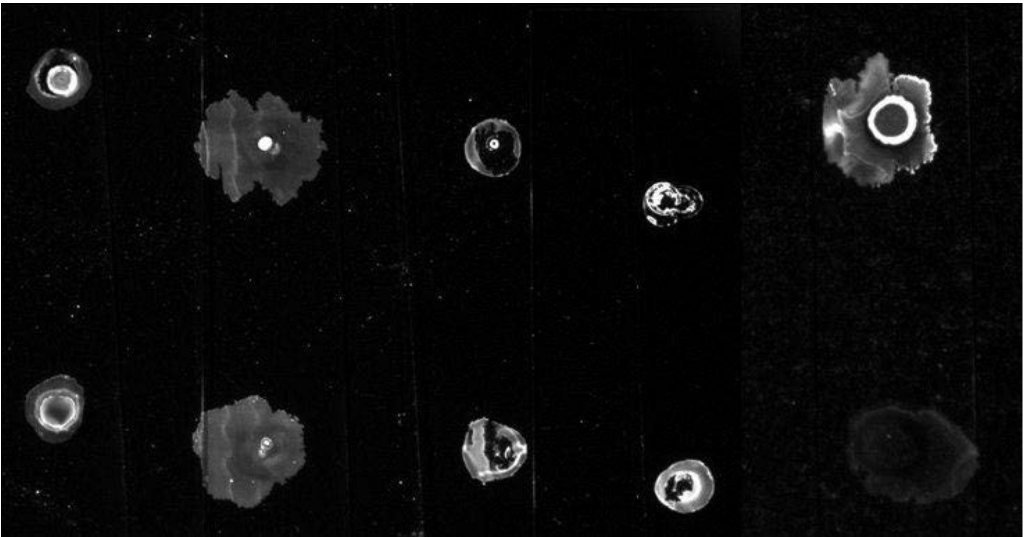


Figure 3-16: Amino acid standards (0.1 M, top, 0.01 M bottom) on glass slides developed with aptamer 2 at 0.05 mM concentration. From left to right: His, Ser, Gly, Ile, Phe. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

In initial testing on latent fingermarks the aptamer reagents did not visualise any ridge detail at all. This was seen for both natural and charged fingermarks. Only very strong fluorescent stains were visible on the glass slides after application, by pipette, of the reagent to the fingermark (Figure 3-17). The lack of ridge detail development seems due to the aptamer reagent being too overpowering with the very strong fluorescence of the dye.

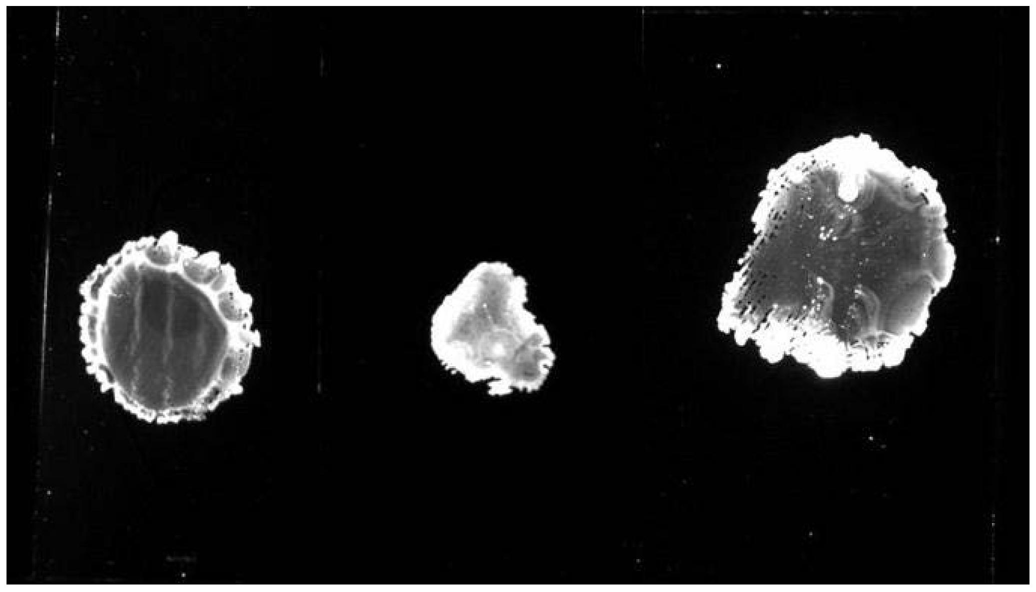


Figure 3-17: Charged (left), natural (centre), and eccrine groomed (right) fingermarks after treatment with the aptamer 2 reagent at 0.1 mM concentration. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

In order to try to improve these initial results, a wash step was investigated. The idea of the wash step was to remove the excess stain which was overpowering the fingermark but at the same time leave behind any possible ridge development. The first wash step trialled was the solvent solution for the aptamer reagent. A methanol:PEG solution at a PEG concentration of 10 µg/µL was used. This solution was used as it was already known that the solution would dissolve the stain but it was thought that the amino acids in the fingermark detail would not be removed due to the lower polarity of the solution. The wash step was carried out after recording initial visualisation with 75 µL of the wash solution being run over the slide before being left to dry. When tested initially on amino acids, the wash worked very well, removing all of the excess stain and leaving the fluorescent interaction with the amino acid intact and clearly visible as seen in Figure 3-18.

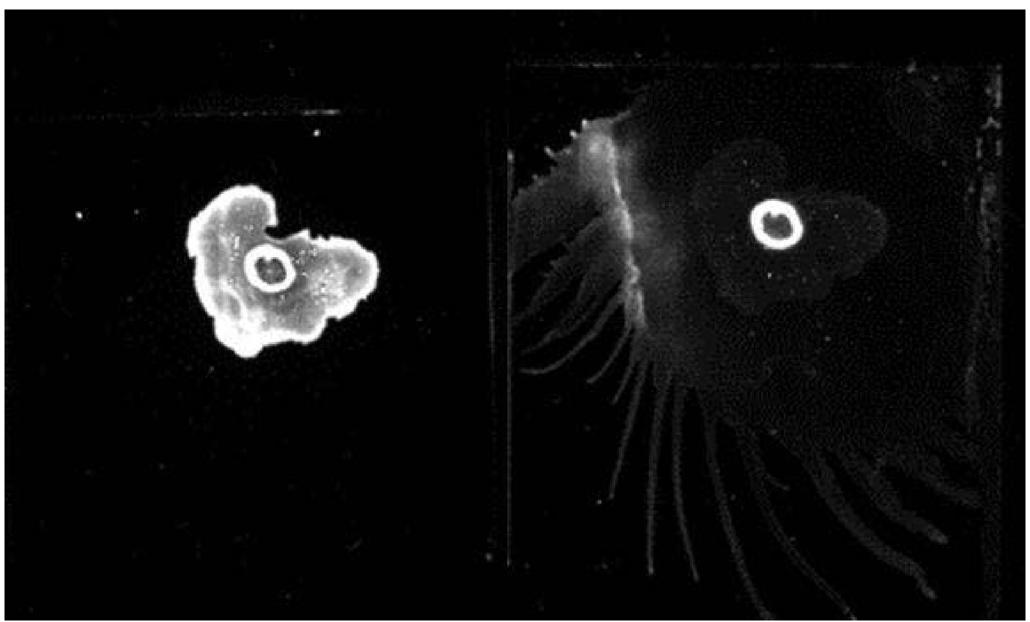


Figure 3-18: Histidine (0.1 M) after development with aptamer 1 at 0.05 mM concentration (left), and after washing with methanol:PEG (right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

With this wash step, however, it soon became apparent that it was unsuitable for use on latent fingermark samples. The wash solution was applied to the fingermark samples in exactly the same manner as for the amino acid spots; however, when visualised, there was no improvement in detail, with the stain just being further dispersed across the slide (Figure 3-19). This was believed to be due to the diffusion of both the lipophilic and hydrophilic fractions.

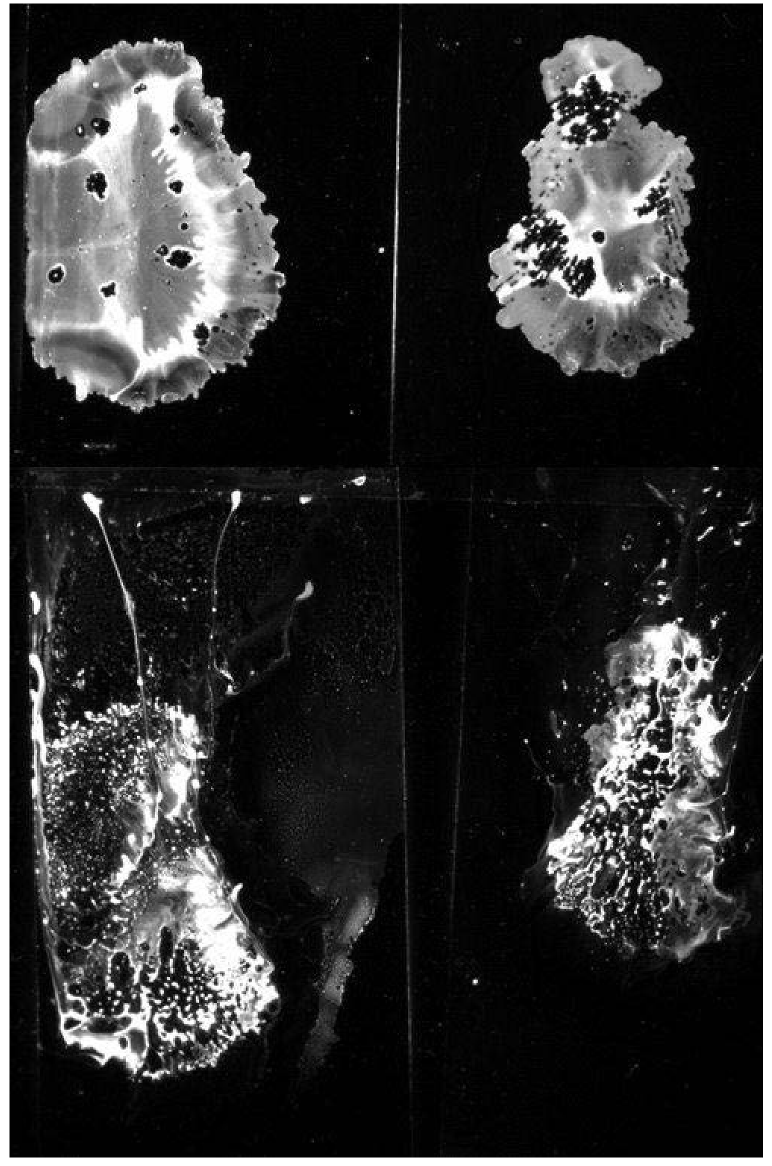


Figure 3-19: Natural (left) and charged (right) fingermarks after development with aptamer 1 at 0.1 mM concentration (top). Then, after washing with methanol:PEG (bottom). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

With this initial post-development wash solution washing away everything including any possible developed ridge detail, a second wash step was developed that was less polar and hence less vigorous in washing away the detail of the latent mark. A wash solution of acetone and methanol in a 90:10 v/v solution was developed and was found to produce better detail than had been previously achieved. The wash step appeared to not only remove the excess reagent but also allow for re-incubation of the reagent which in turn produced greater development of the fingermark ridges. It was also found that a reagent with a much lower concentration of aptamer was required for ridge development. Detail was much clearer when

the aptamer was used at a concentration of 2.5 µM (Figure 3-20). A concentration of 0.05 mM was also possible for successful visualisation but it required several washes to produce acceptable detail (Figure 3-21) and therefore wasted reagent.

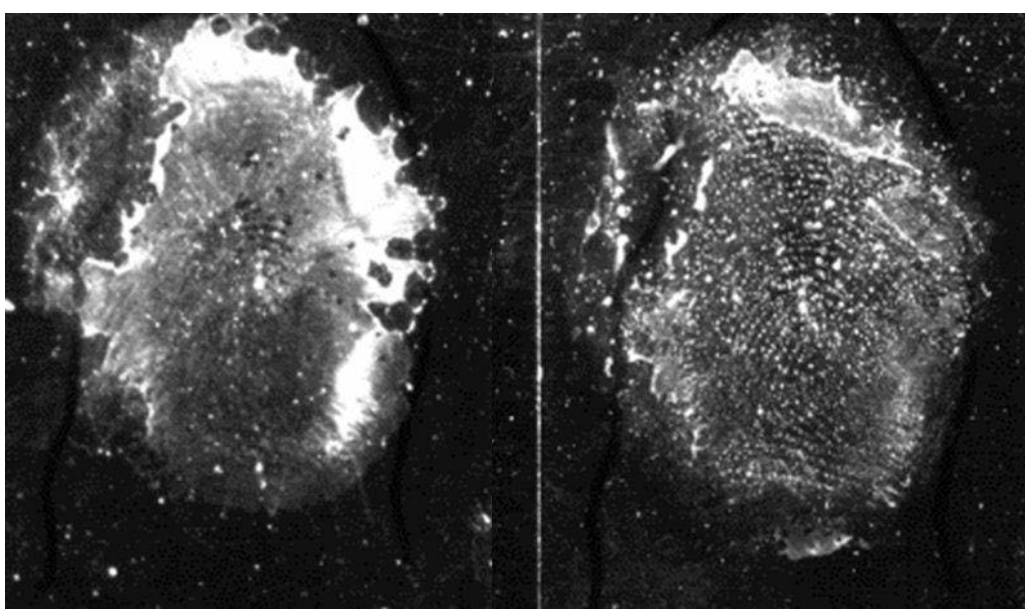


Figure 3-20: Eccrine groomed mark developed with aptamer 2 at 2.5 µM concentration (left), and after washing with acetone:methanol (right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

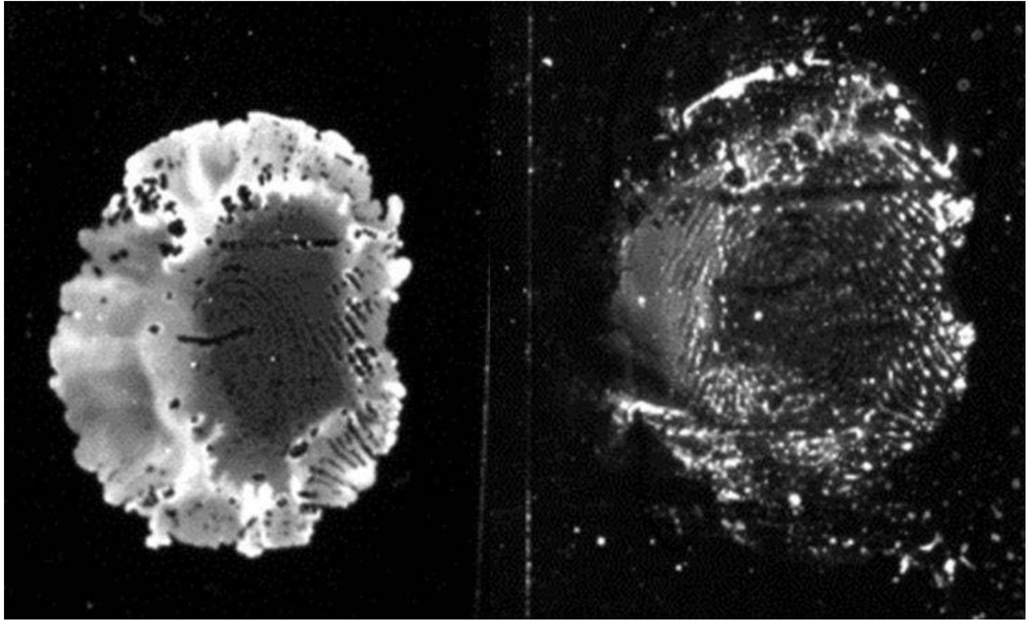


Figure 3-21: Eccrine groomed mark developed with aptamer 2 at 0.05 mM concentration (left), and after washing with acetone:methanol (right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

The reason for why a much lower concentration of aptamer is required for the development of ridge detail is likely due to the fact that the aptamer reagent was too fluorescent and obscured any detail visible at the higher concentrations. With a lower concentration, there is less aptamer available and therefore the reagent is directed more to the ridges and the amino acids rather than excess being applied and the reagent sitting over the whole mark.

The use of an adapted art-craft airbrush as a secondary method to deliver the aptamer reagent was also investigated. Although the pipette method was able to produce results, application was quite difficult with the reagent generally being unevenly applied. It also had the potential for damaging the latent fingermarks with the pipette tip as this had to be kept close to the glass slide to be able to sufficiently apply the aptamer reagent over the entire mark area. It was found that the airbrush required a minimum of 60 µL of the reagent to be added to the reservoir for sufficient application over the entire fingermark sample. Although this was three times the amount of reagent than had been used with the pipette method, it was found that the method on the whole produced greater development than had been achieved previously. All marks tested with the airbrush method were done with the aptamers at a concentration of 2.5 µM due to the volume of reagent that had to be used and the limited amount of aptamer initially available.

Generally, it was found that, with the spray method, natural and eccrine groomed marks produced clearer detail than those obtained from charged marks (Figure 3-22). This may be due to the reduced incubation time that resulted with the airbrush technique. Although more reagent was applied to the slide, it was found that the reagent dried far quicker when applied with the airbrush than when applied with a pipette. This therefore reduced the incubation time the aptamer had with the amino acid, which could be why the charged marks were less developed as amino acids are known to be contained within a charged fingermark emulsion created with the sebaceous material.

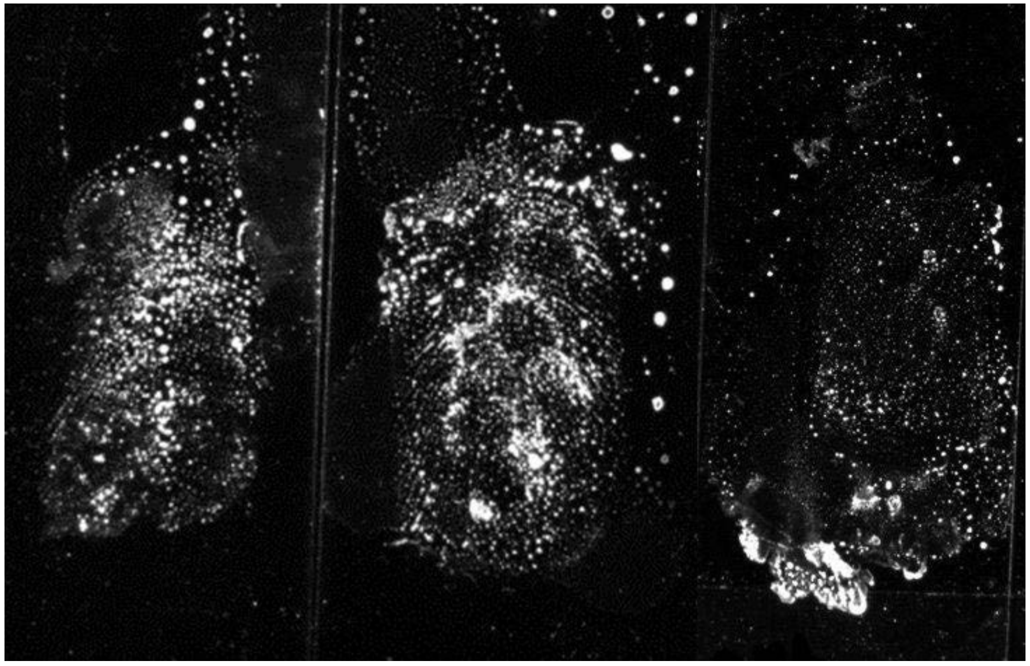


Figure 3-22: A natural (left), eccrine groomed (centre) and charged (right) fingermark after development with aptamer 1 at a 2.5 µM concentration. Application of the reagent was with an airbrush. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

In terms of the quality produced by the two different aptamers, both were found to behave similarly with typical results as presented in Figure 3-22. At this point in the study, further optimisation was undertaken to try to improve the results. It was during this optimisation period when some results were re-run that it was found that there may be some non-specific interaction occurring between the aptamer reagent and the amino acids/fingermark deposits. At this point, no further investigation into the aptamer reagent was undertaken while the level of non-specific interaction was determined. This is discussed in Section 3.5.4.

3.5.3 Suitability of the Rofin Poliview for visualisation

As detailed in Section 3.3.2, the dye chosen to provide visualisation was hexachloro-6-carboxyfluoescien. This dye was chosen as its excitation and emission properties were similar to those of IND-Zn making it an ideal dye for comparison studies to existing techniques (Figure 3-23 and Figure 3-24). The dye was also chosen as it would allow results to be visualised using available imaging systems.

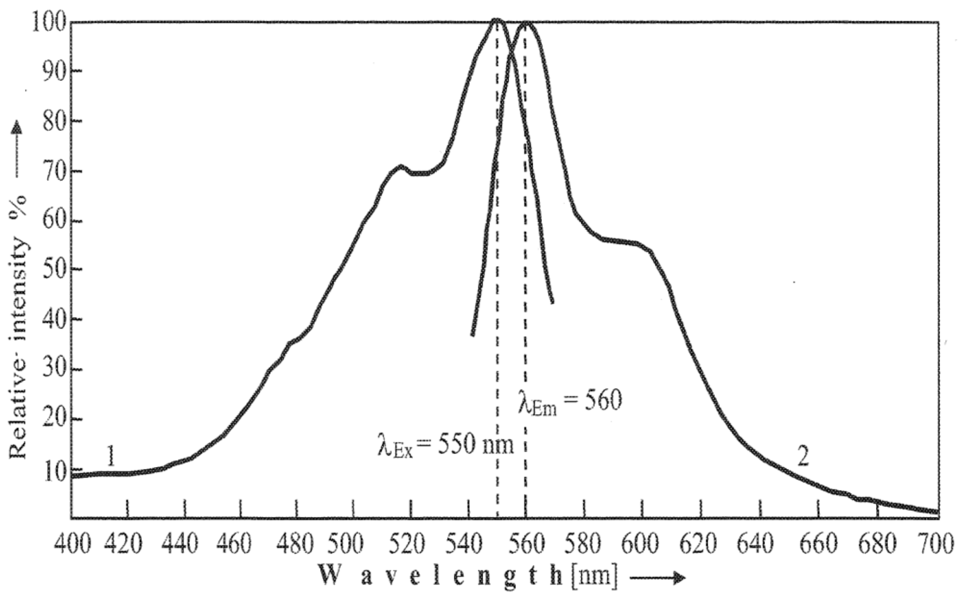


Figure 3-23: Luminescence spectra for a latent fingermark developed with IND-Zn. Excitation (1), Emission (2). Taken and reproduced from Stoilovic and Lennard [225].

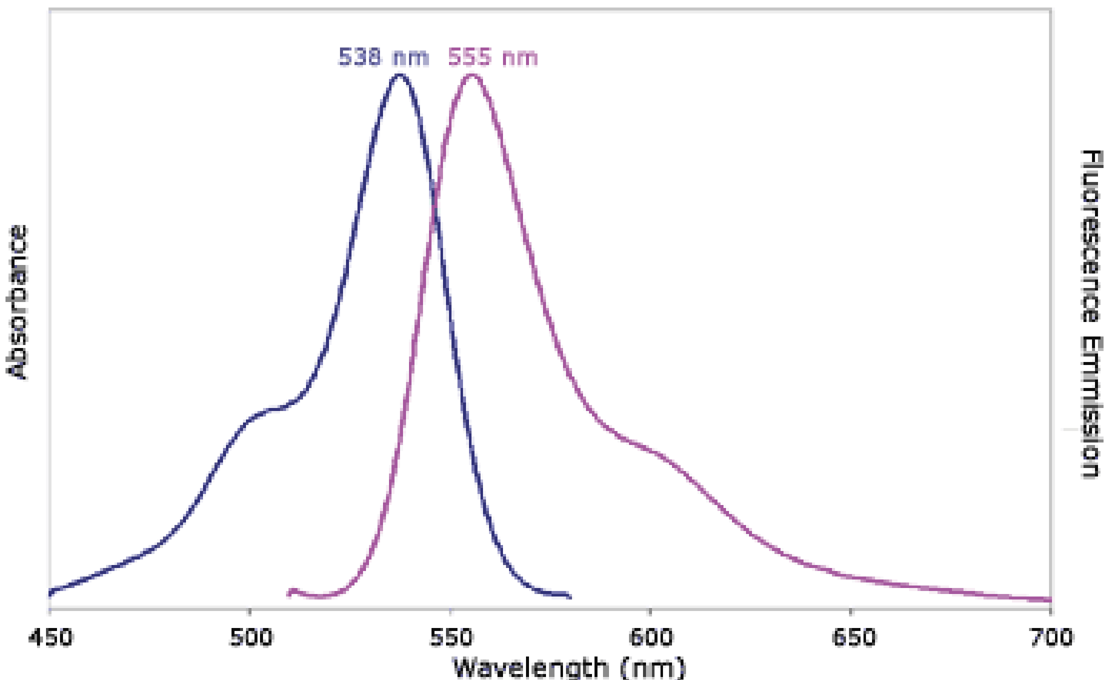


Figure 3-24: Luminescence spectra showing excitation and emission maxima for hexachloro-6-carboxyfluorescein. Taken and reproduced from Integrated DNA technologies Inc. [223].

There were two imaging systems available in this study, these being the Rofin Poliview IV and the Foster and Freeman video spectral comparator (VSC) 6000. Although most indanedione development in our laboratory is visualised using the Poliview, with an excitation band-pass filter of 505 nm and a camera band-pass barrier filter of 555 nm, it was decided that the VSC should be investigated as a potential alternative for visualisation of the aptamer reagent.

With the given spectra of the HEX dye, the most suitable settings on the VSC for optimum visualisation were determined to be excitation with a band-pass of 485 – 535 nm with a 550 nm long-pass barrier filter for observation. With these settings, it was found that very intense fluorescence was achieved even when the reagent was applied at lower concentrations (0.05 mM). The intense fluorescence obtained therefore meant that short integration times of 8 ms were generally sufficient for visualisation. However, the major drawback with the images obtained using the VSC were that they generally appeared grainy which made it difficult to see all of the development; this would be a major problem when dealing with the fine detail of latent fingermarks (Figure 3-25). These grainy images were later found to be due to a software bug in the auto-averaging feature of the software.

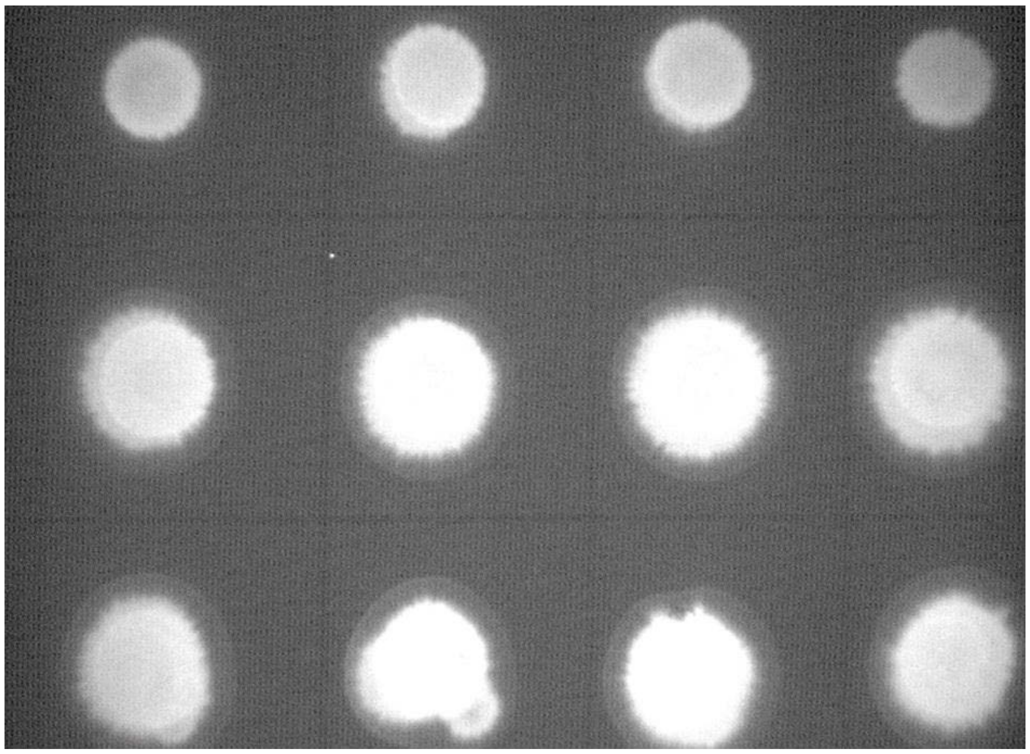


Figure 3-25: Amino acid standards developed with aptamer 1 at a 0.05 mM concentration. Image recorded in the fluorescence mode on the VSC using a 485-535 nm band-pass filter for excitation, with a 550 nm long-pass camera filter.

When the same samples were viewed on the Poliview, however, it was found that images of much greater clarity and detail were produced (Figure 3-26). The optimum settings for visualisation of the aptamer reagent were found to be exactly the same as those used for IND-Zn. A band-pass filter of 505 nm with a 40 nm band width was used for excitation while a camera barrier filter of 555 nm with a 10-20 nm band width was used for visualising the emission. These settings were tested several times with various concentrations and surfaces and all produced images which were clear and detailed. This work therefore showed that the Poliview system would be very suitable as the imaging system to use with these reagents.

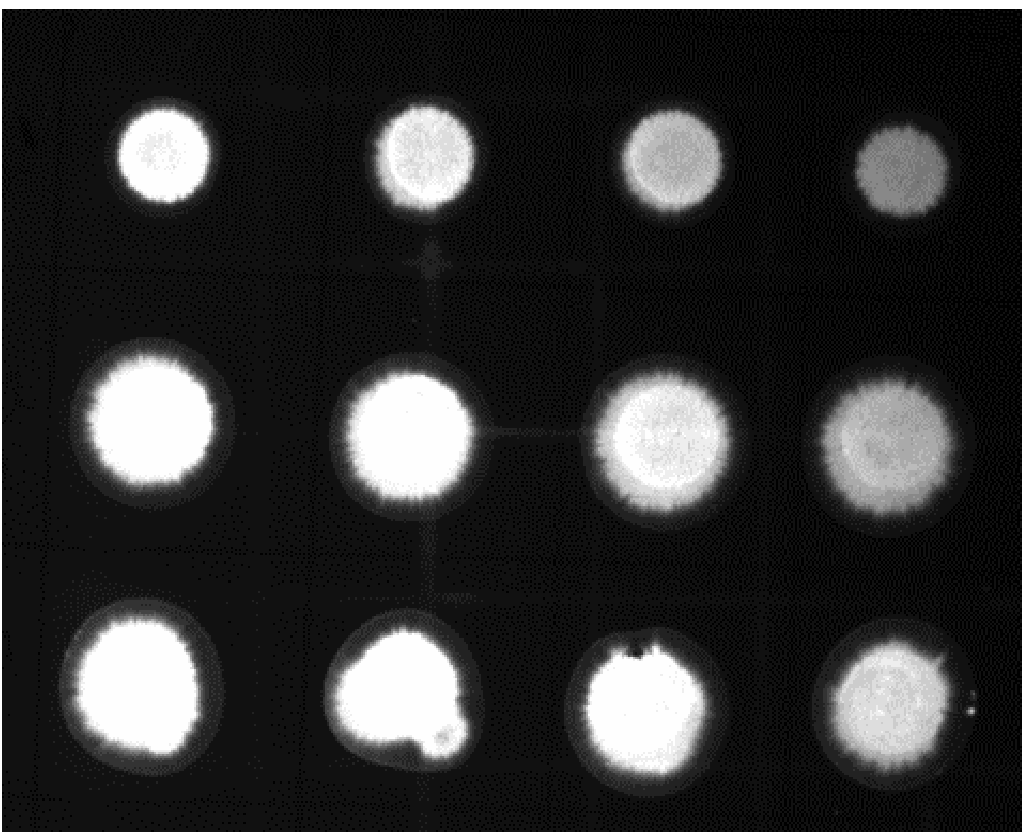


Figure 3-26: Amino acid standards developed with aptamer 1 at a 0.05 mM concentration. Image recorded in the fluorescence mode on the Poliview system using a 505 nm excitation band and a 555 nm band-pass camera filter.

3.5.4 Identification of non-specific binding

Non-specific binding experiments were conducted to investigate if the aptamer reagents were showing any affinity towards other fingermark components other than the amino acids. This was done by using a modified ELISA approach. ELISA is a very common biochemistry

assay used to determine the presence of a target molecule or to study the binding affinity and specificity of antibodies. By using antibodies and enzymes, the specific detection of a wide variety of targets can be undertaken. The general concept was to add various targets individually to an ELISA plate and test them against the aptamer reagents. After several washes, any aptamer interaction with the targets would be visible by examining the plate for remaining fluorescence therefore highlighting aptamer affinity.

The targets tested in this modified approach were the amino acids L-histidine, L-isoleucine, L-serine, L-phenylalanine and glycine. Also tested were four components commonly found within latent fingermarks, which were thought to be potential targets for the aptamer reagent due to their larger molecular size compared to amino acids. Those chosen were glucose, palmitic acid, oleic acid and squalene.

Using an ELISA plate, several rows were filled with targets and allowed to incubate, which allows the target to adhere to the plastic walls of the plate through charge interactions. The wells were then washed to remove excess target before the aptamer reagent was added to incubate with the remaining target in the well. Excess reagent was then washed away and the plate visualised in the luminescence mode for any potential binding.

Unfortunately, this approach was not able to provide any information regarding aptamer interaction as shown in Figure 3-27. After incubation and washing of the reagent, none of the wells remained fluorescent. Several attempts at this method were tested including stronger concentrations, longer incubation times and less wash steps but all were unsuccessful. One reason for this may be due to the acetone:methanol wash used. Water as a wash was initially tested but this had also given negative results which were thought to be due to the re-solubilisation of the amino acids. The acetone:methanol wash was therefore used as it had been successful as a wash with developed amino acids and fingermarks on the glass slides developed with the aptamer reagents. However this wash may have caused the re-solubilisation and removal of the targets by interfering with the charge interactions between the targets and the plastic wells.

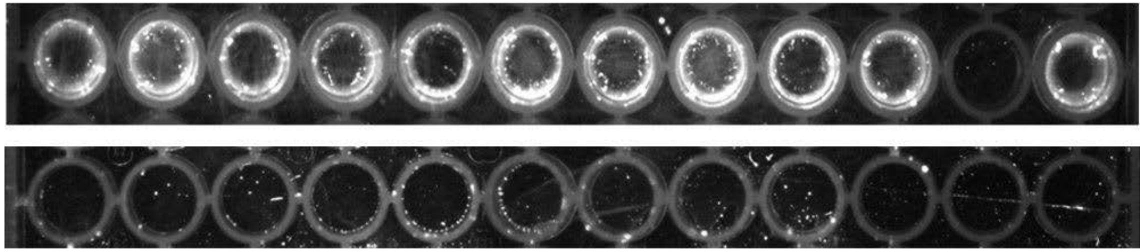


Figure 3-27: ELISA plate during incubation with targets and aptamer reagent (top) and after washing (bottom). Targets from left to right: L-histidine, L-isoleucine, L-serine, L-phenylalanine, glycine, glucose, palmitic acid, oleic acid, squalene, water, blank, and aptamer reagent. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

A second test to investigate non-specific binding was carried out due to the potential interaction of the HEX dye tag with the amino acids and latent fingermarks. Although, initially, amino acid and fingermark detection seemed to be occurring through aptamer interactions in the reagent, further testing provided conflicting results. During further optimisation studies, both aptamer sequences were re-run against all previously tested amino acid spots. During this, it was found that, instead of the aptamer sequences just showing binding to certain dried amino acid spots as seen previously (see Section 3.5.2), the aptamer sequences now showed binding to all the amino acids regardless of the aptamer reagent used.

Because of this, a trial was run to compare results obtained from the aptamer reagents compared to those obtained with a reagent that consisted of just the dye in solution. The HEX dye (Figure 3-28) itself was not available for purchase without the attachment to an oligonucleotide; therefore, a similar dye had to be sought. 5(6)-Carboxyfluorescein (FAM) (Figure 3-29) was identified as the closest related compound available for purchase. This molecule is analogous to HEX and is the most commonly used fluorescent derivatization reagent for the labelling of biomolecules [236].

Figure 3-28: Structure of hexachloro-6-carboxyfluorescein (modified from Integrated DNA technologies Inc. [237]).

Figure 3-29: Structure of 5(6) carboxyfluorescein (modified from Santa cruz biotechnology[238]).

Before this dye was used in non-specific testing, the dye had to be tested for suitability with the Poliview system and settings previously used as the absorbance maximum (495 nm) and emission maximum (520 nm) were lower than those of the HEX dye (Figure 3-30). However, through comparisons at various concentrations, it was found that the FAM dye was able to provide the same intense fluorescence as seen with the oligo-attached HEX dye.

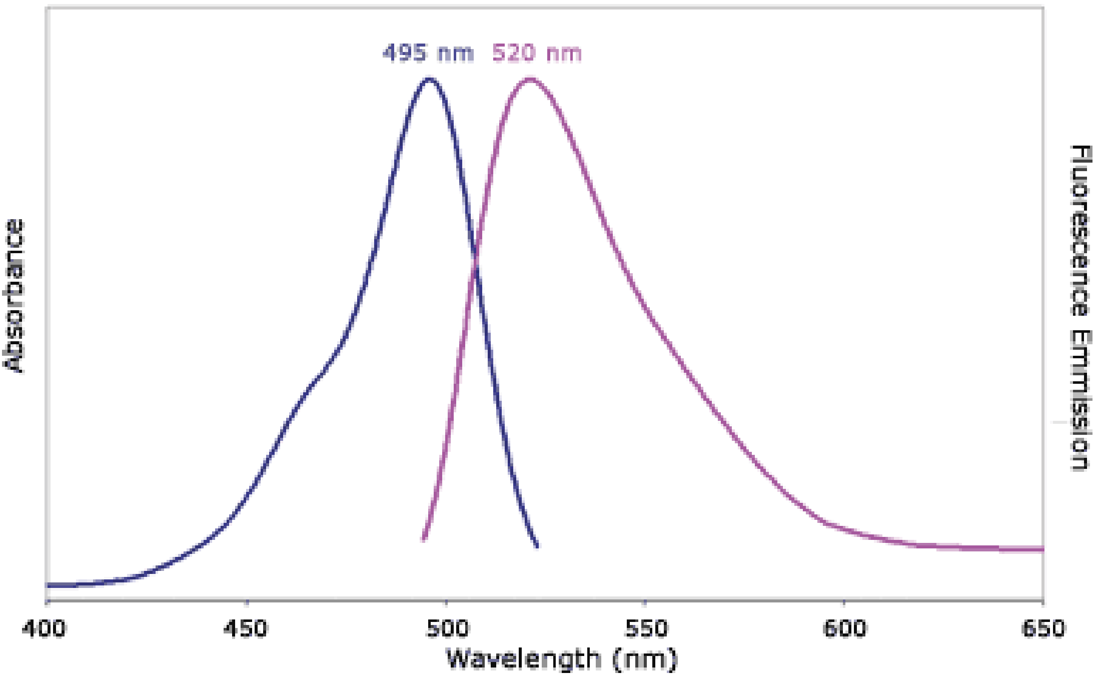


Figure 3-30: Luminescence spectra showing excitation and emission maxima for 5(6) carboxyfluorescein. Taken and reproduced from Integrated DNA technologies Inc. [239].

The FAM dye was dissolved in methanol to create a stock solution of 0.5 mM. PEG was added to this solution at the same ratio as had been used when dissolving aptamer 2. This stock solution was then diluted further with methanol to the same concentrations used with the aptamer reagents (0.05 mM & 2.5 µM). First, comparisons were undertaken using the aptamer reagents and the FAM solution on amino acid standards (0.01 M) dried onto glass microscope slides. Results from these tests showed some striking similarities between amino acids developed using either aptamer reagent and the FAM solution (Figure 3-31). Results were very hard to distinguish, with all tests generally producing a strong fluorescent outer

circle where the solvent front and fragments of the dried amino acids had reached, with an inner fluorescent spot highlighting where the remaining amino acid had dried.

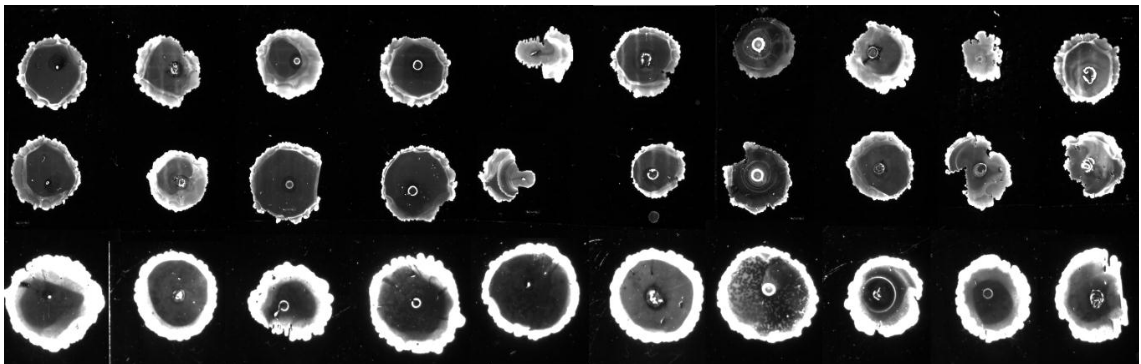


Figure 3-31: Amino acid standards developed with aptamer 2 (top), aptamer 1 (middle) and FAM (bottom), all at a reagent concentration of 0.05 mM. Amino acids (0.01 M) from left to right: Gly, Ile, Thr, Ala, Ser, Leu, Lys, Val, His, Phe. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

Comparisons were then run against latent fingermarks placed onto glass microscope slides. Both charged and natural fingermarks were treated with the FAM solution and the aptamer reagents. Split fingermarks were used to allow one side to be developed with the aptamer reagent while the other side could be developed with the FAM solution, allowing for a precise comparison across the same mark. Fingermarks were developed using the spray technique with each development requiring a post-development wash in the acetone:methanol wash as previously described (see Section 3.4.4). It was found that, with each reagent, a concentration of 2.5 μM gave the greatest detail and this was used throughout the investigation.

On both natural and charged fingermarks, it was found that in general the development was very similar between the aptamer reagent and the FAM solution (Figure 3-32). Although, on the whole, fingermark detail was poor with either technique, results were able to provide some ridge outlines and general fingermark patterns. Again, it was seen that natural marks provided development that were greater in clarity and intensity than those obtained from charged marks. This was true of both the aptamer and FAM reagents, highlighting that both reagents may prefer direct access to amino acids, rather than being mixed within a latent deposit as is the case with the charged marks (where the amino acids tend to be in an emulsion with the sebaceous components).

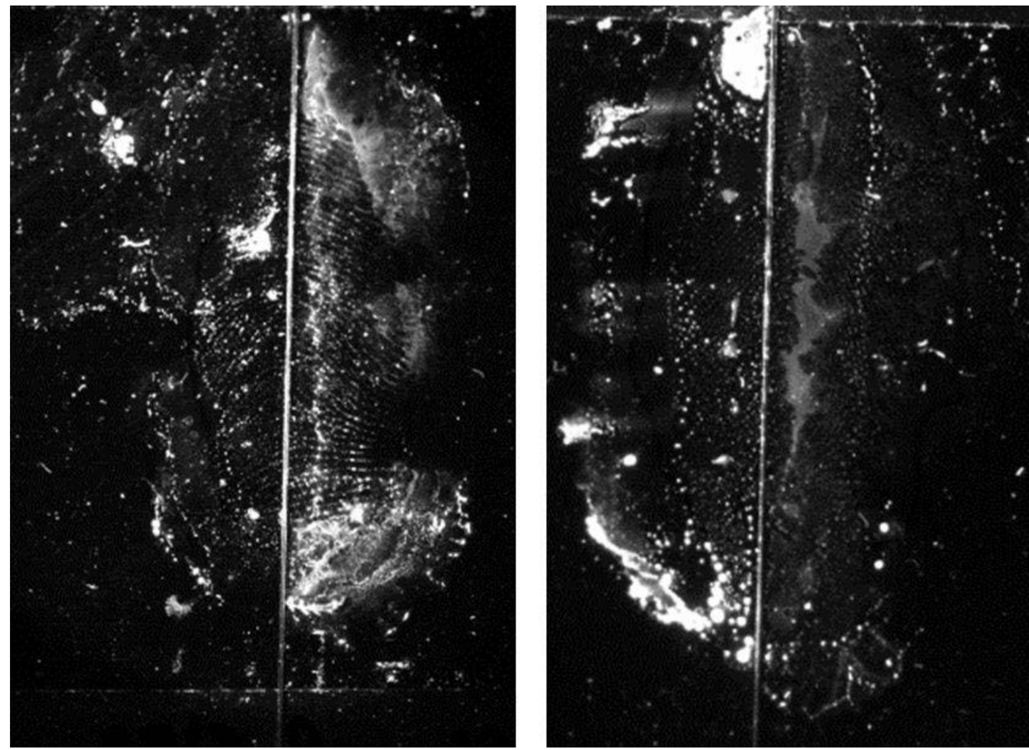
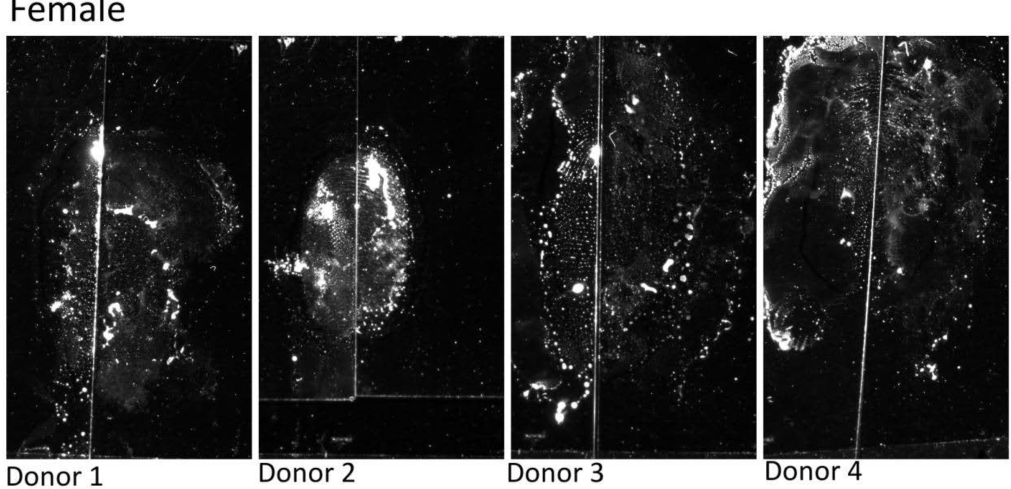


Figure 3-32: Natural fingermark (left image) and charged fingermark (right image). Both fingermarks were developed with FAM (left side) and aptamer 2 (right side) at a 2.5 µM concentration. Both fingermarks were then washed in acetone:methanol for 15 seconds. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

These results were backed up with results obtained using natural and charged fingermarks from eight donors of both male and female sex. Donors placed split marks on glass slides which were then developed with aptamer 2 or the FAM reagent. Again, results tended to follow the same trend although, with the donor marks, results varied from no development at all to almost full ridge detail being visible (Figure 3-33 and Figure 3-34). These results can be explained by the huge variation that occurs in latent fingermark composition between donors, with the reagents only interacting with certain fingermark components, those being mainly amino acids.



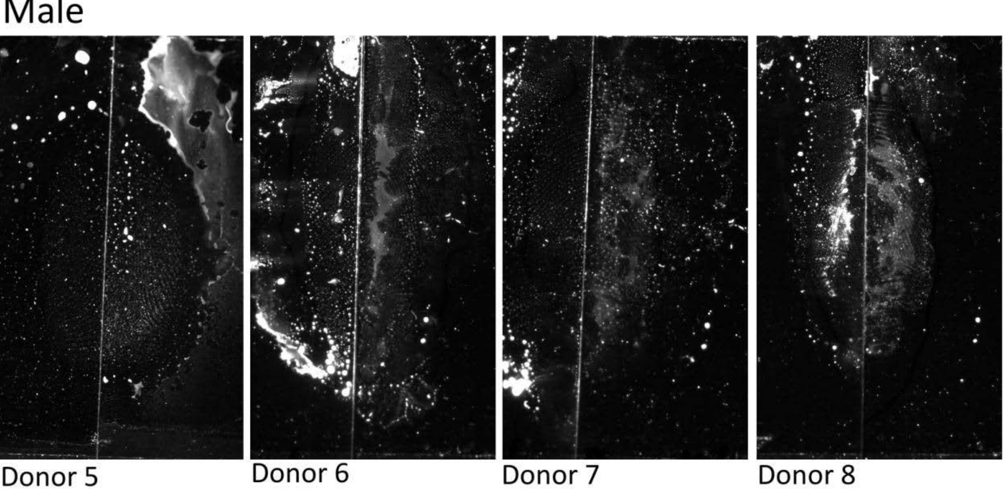
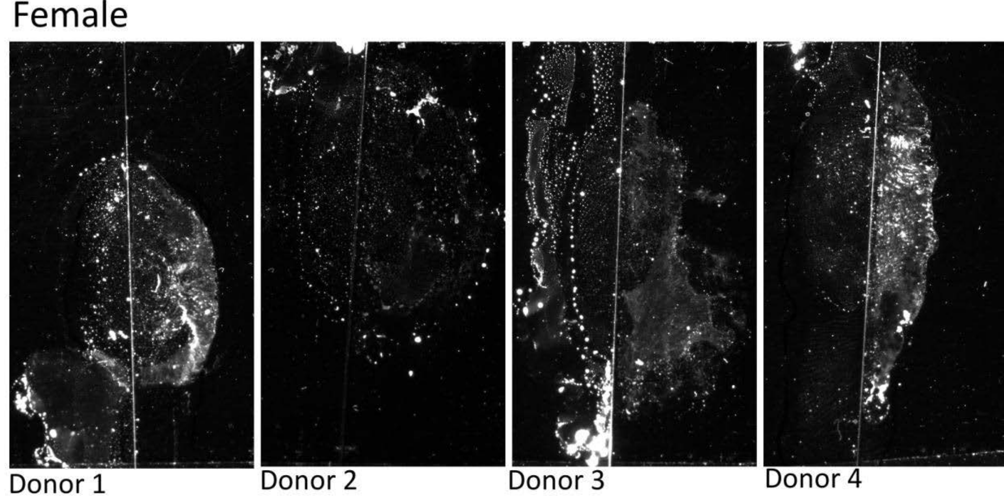


Figure 3-33: Natural split fingermarks from 4 female and 4 male donors. All marks were developed with FAM solution (left side) and aptamer 2 reagent (right side). All treated marks were subsequently washed with acetone:methanol for 15 seconds. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.



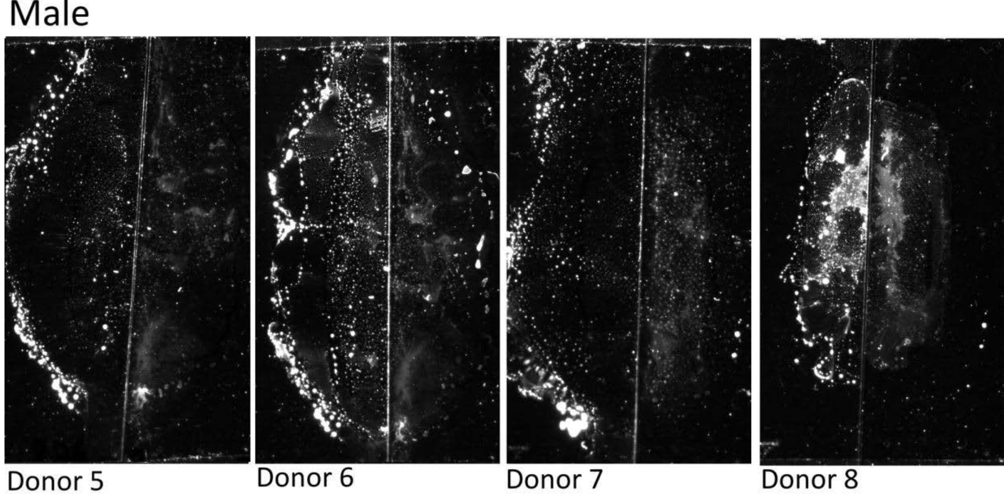


Figure 3-34: Charged split fingermarks from 4 female and 4 male donors. All marks were developed with FAM solution (left side) and aptamer 2 reagent (right side). All treated marks were subsequently washed with acetone:methanol for 15 seconds. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

With these results, it is clear to see that there is an interaction to some extent occurring between the dye attached to the aptamer and the amino acids and latent fingermarks. When selecting the most suitable dye for this study, the main factors considered were its compatibility with existing forensic imaging techniques and its potential for comparisons with current fingermark detection techniques. The structure of the dye itself was not investigated due to the location of the dye attachment on the aptamers. The dye was attached at the 5´-end, an area which is away from the binding section of both aptamers. With this it was thought that the binding affinity of the aptamers for amino acids would be greater than any

non-specific dye-secretion interactions. As can be seen in the structure of both HEX and FAM, there are a number of sites that would allow for hydrogen bonding to occur. It is believed that this is the reason behind the dye interactions, with hydrogen bonding occurring between the amino acids in the latent fingermarks and the dye molecule.

3.6 Conclusions

This study set out to determine whether an RNA aptamer-based reagent could be used to detect and visualise latent fingermarks. Aptamer-based reagents were developed by commercially synthesising aptamers that had been published in research to bind to various amino acids. These aptamers were modified with the addition of a fluorescent tag (HEX), which was chosen due to its fluorescence properties that resemble those of the commonly used fingermark reagent IND-Zn. The reagents were tested for affinity to both amino acid standards and latent fingermarks placed on glass microscope slides and were visualised using existing forensic imaging systems.

Unfortunately, the potential of the aptamer-based reagents could not be fully investigated due to non-specific interactions mainly occurring between the fluorescent tag and the amino acids. It was found that the dye employed was able to bind to amino acids without being attached to the aptamers. Through further research, it is believed that the reason for this was that there were hydrogen bonding interactions occurring between the dye and the amino acids. Further issues were identified when attempting to use the aptamer-based reagents on porous surfaces. Initially, the reagents were designed for use on porous surfaces but another non-specific interaction was found between the aptamer and the cellulose surfaces. When the RNA aptamer-based reagent came in contact with the cellulose surface, it became strongly attached and was unable to be removed with a number of wash steps. It is believed that this is again due to hydrogen bonding interactions happening between the hydroxyl groups on the RNA phosphate backbone and the cellulose substrate.

This study was not successful in identifying the possibility of an aptamer-based latent fingermark reagent; it did, however, provide a number of answers and solutions to carry forward in the project. A principal realisation from this study was that the aptamers used were not of the correct affinity levels. In research, they had been shown to successfully bind to amino acids and demonstrate very high levels of selectivity however, when these binding

affinity levels are compared to other aptamers that target larger molecules such as viruses and proteins, they are at least 1000 fold weaker. The use of aptamers with stronger binding affinities is clearly needed when aptamers are being used in alternative situations away from the ideal selection and physiological conditions under which they are found to be highly successful. This concept was further strengthened in personal correspondence with Dr. James, an expert in aptamers, who stated that aptamers with affinity levels in the nanomolar range should be used for any detection type experiments [240]. Although this would be the next logical step to follow in this study, the lack of any published aptamers that target amino acids in this range and the fact that new aptamers could not be specifically selected in this project meant that this would not be possible.

After further consideration and as detailed in published research, the use of aptamers to low molecular weight targets when conjugated within a complex matrix such as a protein surface will have great difficulty in recognising the actual target. This is because either the target is hidden within the conjugated compound, or, its three-dimensional structure is not fully accessible for aptamer binding due to the various side chains found on proteins or the natural charges of the protein masking the target [240, 241]. With this, it can be seen that amino acids buried within a complex latent fingermark deposit will most likely cause problems for any aptamer trying to recognise low molecular weight amino acid targets.

Chapter 4: DNA aptamers to lysozyme and fingermarks on PVDF

Chapter 4: DNA aptamers to lysozyme and fingermarks on PVDF

4.1 Introduction

4.1.1 A change of direction

Although results were poor and disappointing, the research with amino acids in the previous chapter (Chapter 3) did provide a plethora of information on the development of an aptamer-based latent fingermark reagent. With this it was decided that, instead of continuing to try and target amino acids, the project should focus on identifying other fingermark components of choice for an aptamer approach. The targeting of other components would allow for a more detailed and thorough understanding of how aptamers might behave when used for this application. With the realisation that amino acids may be too small for the available amino acid targeting aptamers, it was decided that fingermark components of larger molecular size should be investigated. This change of direction allowed for the use of aptamers with far greater binding affinities (low nanomolar range) and also a change from RNA-based aptamers to DNA-based aptamers.

4.1.2 Protein suitability for fingermark detection

As already stated in Chapter 1, latent fingermarks are composed of a complex emulsion of organic and inorganic compounds including many fatty acids, amino acids and over 400 polypeptides [29, 30, 242]. These polypeptides are generally secreted by the eccrine glands and hence can be present in latent fingermarks to some extent. Due to the huge complexity and variance in protein secretion, no full study has been undertaken to investigate the exact proteins and concentrations found within a latent fingermark. Several smaller studies have, however, managed to investigate certain proteins [32, 233, 243, 244].

A number of studies have looked into identifying the major proteins found within eccrine sweat. Nakayashiki identified five main eccrine sweat proteins, those being albumin, transferrin, orosomucoid, Zn-α_2-glycoprotein and lysozyme [245]. Albumin, transferrin and

fast-migrating γ-globulins were detected by Uyttendaele *et al*., in sauna-induced eccrine sweat [246]. While albumin, transferrin and immunoglobulin-G were detected in eccrine sweat by Herrmann and Habbig [247]. The exact amount of protein content found in eccrine sweat has been determined to range between 0.3 – 1.12 g per litre, highlighting the minute protein concentration secreted in human sweat [248].

By using FTIR and FTIR imaging, a number of studies have been able to provide possible characterisations of proteins found within latent fingermarks, such as protein-containing skin cells, however none of these studies have been able to accurately identify or quantify them [249-253]. In a recent study, two proteins within latent fingermarks have been identified with the use of MALDI-MSP, those being dermcidin and psoriasin but again their concentrations were not determined [39].

Although the protein content of latent fingermarks is very low [233], there have been two very successful studies that have used different proteins as targets for latent fingermark detection and visualisation. In 2008, Reinholz used the plasma protein albumin as the target to develop latent fingermarks on six porous materials including recycled paper, white envelope paper and nitrocellulose [128]. By targeting this protein, marks were able to be developed with tertiary level detail up to 130 days after being placed. Proteins involved in the skin's desquamation and antibacterial processes (dermcidin, keratin 1 & 10, cathepsin-D) have also been found by Drapel *et al*. to be successful when used as targets for latent fingermark detection on PVDF and whitened paper, producing clear level 2 and 3 detail [38] (as seen earlier in Figure 1-16).

With these two studies providing results of secondary and tertiary level detail, it can be seen that proteins are viable as targets for latent fingermark detection techniques. Like amino acids, proteins generally have a strong affinity for cellulose and therefore remain intact and stable on porous surfaces. To date, however, proteins remain an underutilised target for latent fingermark detection, with the majority of detection and visualisation techniques relying on either amino acids or sebaceous content (fatty acids, triglycerides) for successful development. However, with the need for greater sensitivity and selectivity with latent fingermark reagents, and with the successful initial use of proteins for detection, it may be that proteins hold the key in providing a transformational change in fingermark detection by providing an additional avenue as a class of target compounds.

4.1.3 DNA aptamers to proteins

As has already been highlighted in Chapter 2, the diversity of aptamers available today is vast. Aptamers have been successfully selected to all types of targets, ranging from small molecular compounds such as organic dyes, amino acids, metal ions and nucleotides, to larger and more complex targets including proteins, viral particles, cell adhesion molecules and pathogenic bacteria [130, 131].

Aptamers to proteins is one area of aptamer research that has been around since the start of the *in vitro* selection of aptamers. Tuerk and Gold in their ground-breaking 1990 paper where they describe for the first time the SELEX procedure used the bacteriophage protein T4 DNA polymerase as the target for an RNA-based aptamer selection [144]. Although this first aptamer to a protein was RNA-based, it was not long until a DNA-based aptamer was selected. In 1992, Bock *et al*. were the first to identify an aptamer to a protein that does not normally interact with DNA or RNA [254]. The protein target was human thrombin, a serine protease enzyme involved in blood clotting. Using ssDNA, Bock *et al*. identified several DNA aptamers that bound to human thrombin with binding affinities between 25-200 nM while also inhibiting the function of human thrombin [254].

Since these early beginnings, the selection of aptamers to proteins has increased dramatically. Proteins have been a staple target for aptamer discovery for numerous reasons. First, proteins are a favourable target for aptamer binding. With extensive surfaces of ridges, grooves, projections and depressions, together with a plentiful supply of hydrogen bond acceptors and donors, proteins provide great opportunities for aptamer interaction [139]. Second, aptamers selected to proteins often bind to functionally important parts therefore inhibiting the protein [255]. With this action, aptamers can interfere with proteins implicated in pathological conditions and can therefore be used as pharmaceutical agents [139]. Aptamers to proteins are also very useful in diagnostic approaches, where aptamers selected to various proteins can be used instead of the more traditional techniques including the use of antibodies that are known to have certain drawbacks as stated in Section 2.4.

As can be imagined, with these benefits for the selection and use of aptamers to proteins, the amount of both RNA- and DNA-based aptamers available to proteins today is substantial and increasing. To list these aptamers is unnecessary for the aim of this introduction; however, an

overview of the diverse range of DNA-based aptamers to proteins is provided. This is shown in Table 4-1.

Table 4-1: DNA-based aptamers to various protein targets and their application.

Protein target	Binding affinity (K_d)	Application	Reference
Enterotoxin of *S. aureus*	2 nM	Biosensor for food poisoning related bacteria	[256]
Campylobacter jejuni	292 nM	Assay based detection	[257]
Mucin 1	0.135 nM	Therapeutic prevention	[258]
Platelet-derived growth factor	50 pmol/L	Therapeutic inhibitor	[259]
Willebrand factor	2 nM	Therapeutic inhibitor	[260]
Bacillus anthracis	200 nM	Electrochemiluminescence sandwich assay	[261]
HIV-1*trans* activation responsive RNA element	20-120 nM	Possible inhibiting of TAR-dependant activation of transcription	[262]
Vascular endothelial growth factor	130-500 nM	Biosensor for cancer diagnosis	[263]
Human RNase H1	10-80 nM	RNase H1 inhibitor	[264]
Thrombin	20 nM	Protein-protein interaction studies	[265]
Lysozyme	2.8-31 nM	Quantum dot biosensor	[266-268]

As table 4-1 shows, aptamers have been successfully selected to a wide range of protein targets. While the application of aptamers has already been discussed (see Section 2.5), it is important to state here the two main uses for the majority of aptamers to proteins. First, highly sensitive biosensors are being developed for use in fields such as medicine to detect and screen for pathogens, viruses and proteins. While, secondly, they are being highly researched for use in therapeutics where they have enormous potential in the fight against two of the world's most deadly illnesses, cancers and the HIV/AIDS virus.

Although there are many aptamers to proteins, the vast majority are selected and developed for use in one of these two main areas of research. Without the use of SELEX (as is the case in this project), this means that, when using published aptamers for alternative approaches such as latent fingermark development, the choice of what aptamers to use is limited to whether there are aptamers selected to the proteins of choice. Lysozyme has, however, been detected in human sweat and, as Table 4-1 shows, aptamers have been successfully selected to this protein. The selection of suitable lysozyme aptamers for latent fingermark detection is described in Section 4.3.1.

4.2 Objectives

The objective of this chapter was to investigate the potential of using DNA-aptamers selected to lysozyme as a possible latent fingermark detection and visualisation reagent. The research in this chapter includes the identification of suitable protein targets within latent fingermarks that already have aptamers selected to them. With results from the previous chapter (Chapter 3) taken into consideration, the research in this chapter needed to look carefully into suitable visualisation techniques and development methods. Although the overall objective of this thesis is for an investigation of aptamer use on commonly encountered fingermark substrates, this chapter focused on using substrates frequently employed in protein detection strategies. By using common biological methods, such as Western blot, it was envisaged that aptamer interactions would be better investigated and understood. With a more biological route followed, the work in this chapter aimed to re-assess the potential of aptamers for use in fingermark detection and ultimately provide a foundation for further research.

4.3 Experimental design

4.3.1 Selection of suitable sequences to proteins in latent fingermarks

As has already been noted (see Section 4.1.2), the precise protein content found within a latent fingermark remains unknown. Only through the research mentioned previously are proteins beginning to be successfully identified. In order to progress further with this project's ultimate aim of investigating the potential of an aptamer-based fingermark reagent,

possible proteins found within latent fingermarks need to be identified. Any proteins identified also need to have aptamers already selected to them due to the limitations of not being able to undertake SELEX selections in this project.

Table 4-2 shows the possible protein targets that could be used for the detection of latent fingermarks. These proteins have been selected due to either their identification in latent fingermarks or as major eccrine sweat proteins that are therefore highly likely to be contained within a latent fingermark deposit to some extent. Given the lack of SELEX capabilities in this project, the table also identifies which of these protein targets have published aptamers selected to them that could be used in this research.

As the table shows, the availability of aptamers to any of the proteins found in sweat or in latent fingermarks is severely limited. There is only one protein that has aptamers currently available, that being lysozyme. Lysozyme was discovered by accident in 1921 by Alexander Fleming when a drop of nasal mucus fell onto a culture plate killing the bacteria completely [269]. Lysozyme is a bacteriolytic enzyme that hydrolyses the *N*-acetylmuramic-β-1,4 *N*-acetylglucosamine linkages of bacterial cell-wall peptidoglycans, breaking down mucopeptides within bacterial cell walls [270].

Lysozyme is widely distributed in human tissue and is present in a number of secretions including saliva, urine, milk and tears. It has been identified in several types of cells including monocytes, macrophages, polymorphonuclear leukocytes and Paneth cells [270]. Lysozyme is present in the skin, being located in the cytoplasm of epidermal cells in granular layers and malpighian cells [271]. Lysozyme is also found in the skin’s pilosebaceous follicle cells, hair bulb cells and all parts of the eccrine sweat gland [216].

Table 4-2: Known or probable proteins found in latent fingermarks and the availability of aptamers to these proteins.

Protein	Locality	Reference	Aptamer availability, type and K_d	Reference
Albumin	Constitutes 60% of serum-protein in healthy humans. Detected as the most abundant protein of eccrine sweat	[128, 245, 246, 272-274]	None available	
Transferrin	Third most abundant protein identified in sweat	[238, 272]	None available	
Lysozyme	Identified as one of 3 main proteins in human sweat. Secreted by the eccrine sweat glands	[216, 245, 270, 275, 276]	DNA sequence, 80 mer, K_d 2.8 nM DNA sequence, 30 mer, K_d 31 nM DNA sequence, 42 mer, no K_d presented	[266-268]
Zn-α_2-glycoprotein	A main protein found in human sweat	[245]	None available	
Orosomucoid	One of 5 main sweat proteins identified	[245]	None available	
Lactate	Reported at significant levels in perspiration (10-40 mM)	[277]	None available	
Dermcidin	Secreted by the eccrine sweat glands. Identified in latent fingermarks	[38, 213, 216, 278, 279]	None available	
Cathepsin-D	Identified as a protein in sweat, secreted by various human tissues. Found in latent fingermarks	[38, 280]	None available	
Psoriasin	Found in epidermis of the face and scalp. Secreted by the sebaceous glands	[215, 281, 282]	None available	
Keratins	Major structural protein of all epithelial cells. Keratins 1 & 10 detected in latent fingermarks	[38, 283]	None available	
Secretory immunoglobulin A	Found at low levels in human sweat	[284, 285]	None available	

Although lysozyme is well known to be present in and on skin, it is only relatively recently that its role has been identified. Lysozyme is classified as an antimicrobial protein (AMP) due to its effectiveness against both Gram-positive and Gram-negative bacteria [216]. It has been found to be secreted by the eccrine sweat glands where, in combination with other AMPs, it provides an efficient innate defence mechanism against many bacteria [275, 286-288]. The amount of lysozyme in healthy human skin samples has been found to range between 75-198 μg wet weight [276]. Its concentration in latent fingermarks, however, is unknown due to the unique and complex nature of latent fingermark deposits as mentioned previously in Section 4.1.2.

All of the lysozyme sequences available are DNA-based aptamers with K_d values in the low nanomolar range. Although there is only one possible fingermark protein available with aptamer sequences, the fact that there are multiple sequences published can allow for comparison studies due to the different lengths and binding affinities shown. Another potential benefit with targeting lysozyme is that it has been shown to be a very favourable target for aptamer binding due to the positive nature of lysozyme [289].

Another encouraging aspect with the selection of lysozyme as the target for this research is that the binding affinities of the lysozyme aptamers are in the region of 1000-fold stronger than those used in the amino acid work. These increased binding affinities are needed as it was seen previously (Chapter 3) that binding affinities in the μM region are not adequate when using aptamers for this application. The size of lysozyme (Figure 4-1) compared to amino acids (Figure 4-2) should also be beneficial when dealing with latent fingermark detection. It was found that, although aptamers had been successfully selected to amino acids, when used in this approach the amino acids became “lost” in the fingermark deposit and the aptamers were unable to bind with them. The larger size of lysozyme together with the increased affinity should allow for the aptamers to successfully bind with lysozyme when contained within the fingermark deposit.

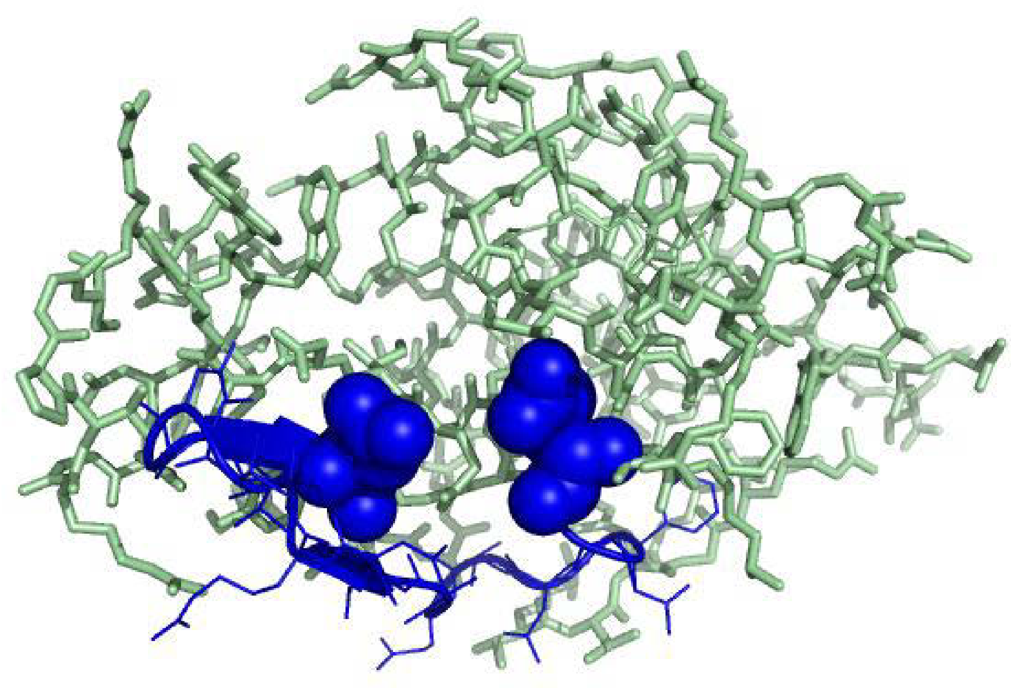


Figure 4-1: A three-dimensional schematic of human lysozyme. The molecular weight of human lysozyme is 14.5 kDa. Taken and reproduced from Griffithbiochem [290].

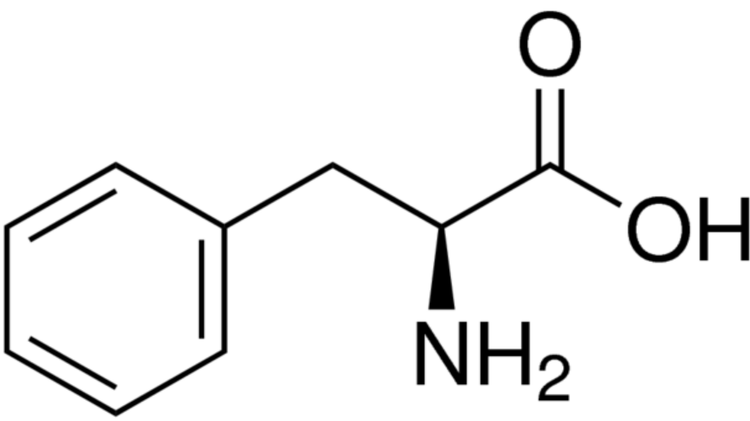


Figure 4-2: Chemical structure of phenylalanine. Phenylalanine is the largest amino acid present in latent fingermarks with a molecular weight of 165 Da. Taken and reproduced from Sigma Aldrich [291].

Of the three aptamers selected to lysozyme, only two were chosen for use in this research. The two chosen were the aptamers selected by Cox and Ellington [268] (30 mer, K_d 31 nM) and by Tran *et al.* [267] (80 mer, K_d 2.8 nM). The third was discarded due to no published data regarding its binding affinity. The two chosen aptamer sequences and data are shown in

Table 4-3. Although having successfully found and chosen two sequences for use in this study, a major problem still arose; both of the aptamers were selected to hen egg-white lysozyme and not human lysozyme. This potential problem, however, was solved with further research surrounding these sequences and laboratory investigations with the aptamers. In further research, it was found that both of the aptamers to lysozyme had been successfully used in various modified biosensors to detect human lysozyme in saliva with good sensitivity [292-294]. Initial investigations with the aptamers in this project also revealed strong affinity to human lysozyme (described in Section 4.3.2).

Table 4-3: Lysozyme aptamer properties.

Aptamer	K_d	Sequence	Predicted secondary structure
Cox (Aptamer 1)	31 nM	ATCAGGGCTAAAGAGTG CAGAGTTACTTAG	None available
Tran (Aptamer 2)	2.8 nM	AGCAGCACAGAGGTCAG ATGGCAGCTAAGCAGGC GGCTCACAAAACCATTCG CATGCGGCCCTATGCGTG CTACCGTGAA	

A major difference with these aptamer sequences compared to those in Chapter 3 is that both lysozyme sequences are DNA-based and not RNA-based. In terms of differences between RNA and DNA aptamers, there is very little to differentiate between them. Both RNA and DNA aptamers share the same characteristics with no difference in their binding mechanisms as both are able to fold into the complex three-dimensional structures needed

for aptamer recognition [295]. RNA is generally preferred in initial SELEX selections, however, due to the greater range of three-dimensional structures produced compared to DNA [296].

In terms of this research, the use of DNA aptamers may be beneficial due to the deoxyribose backbone of DNA. In the work with the RNA aptamers (Chapter 3), it was found that the free hydroxyl group on the ribose backbone of RNA may have interacted with the cellulose substrate, causing the RNA aptamers to become strongly attached to the paper samples. With the deoxyribose backbone of DNA containing no free hydroxyl groups, this interaction can be avoided and thus an interaction with cellulose will be far less likely. The other benefit with using DNA over RNA is that DNA, again due to its deoxyribose backbone, is far more stable than RNA, which is open to degradation through hydrolysis of the free hydroxyl group.

As had been learnt in the previous chapter (Chapter 3), non-specific dye-secretion interactions can occur between the fluorescent tag and the latent fingermark. These interactions were based on hydrogen bonding interactions; therefore, it was necessary to find a dye that would limit these interactions. The tag chosen for this investigation was CAL Fluor® Orange 560, a xanthene dye that fluoresces in the orange region. The structure of this dye is much more suitable for this research as it is far less susceptible to hydrogen bonding than the previously used HEX dye (Figure 4-3). CAL Fluor® Orange 560 was also chosen as it can be used as a replacement for HEX as it demonstrates almost the same excitation and emission properties. CAL Fluor® Orange 560 has an excitation maximum of 538 nm and an emission maximum of 559 nm. It also displays a shoulder on the excitation spectrum at around 500-515 nm (Figure 4-4). It was felt that with this shoulder and the similarity to HEX, CAL Fluor® Orange 560 would be suitable for the forensic imaging systems used in this project.

Figure 4-3: Chemical structure of the CAL Fluor 560 dye and its attachment linker used for the fluorescent visualisation of lysozyme.

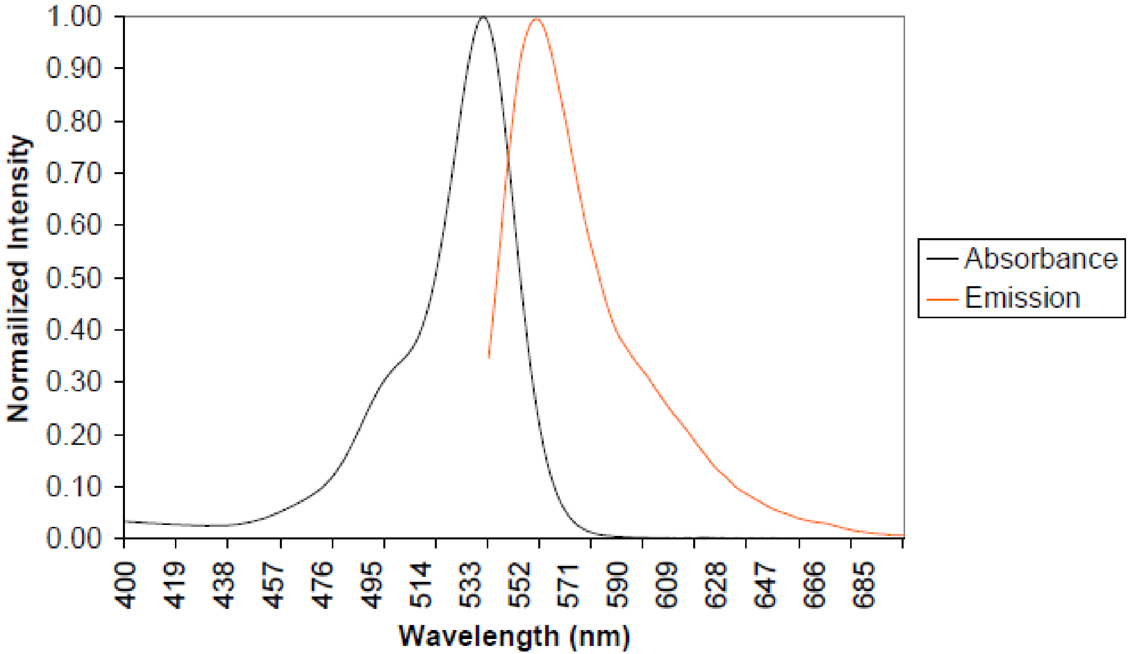


Figure 4-4: Spectra exhibiting the excitation and emission maxima of CAL Flour® 560. Taken and reproduced from Biosearch Technologies [297].

With both the sequences and fluorescent tag selected, the aptamers were commercially synthesised by Biosearch Technologies, Inc. USA. The CAL Fluor® Orange 560 fluorescent tag

was added during synthesis through the reaction of the phosphoramidite dye and the bound monomer, forming a phosphotriester linkage [298]. Both sequences were synthesised on a 1 µmol scale and were purified using RP-HPLC. Again, to allow for numerous experiments to be undertaken with the sequences, each aptamer was divided equally across four tubes. Aptamer 1 contained approximately 516 µg of DNA material per tube while aptamer 2 contained approximately 333 µg per tube. Both sequences were received lyophilised and were stored at 4 ^{O}C until required.

4.3.2 DNA aptamer dissolution and reagent specificity

DNA is highly soluble in aqueous solutions due to its negatively charged phosphate backbone and the high polarity of water. While the general use of water in chemical detection techniques on porous surfaces is impracticable due to amino acids being the target (the exceptions being those that target the lipid content), the use of water as the carrier solvent for protein detection is generally possible due to the poor solubility of dry proteins.

The use of aqueous solutions for the detection of proteins in latent fingermarks using antibodies has already been shown to be successful. Drapel *et al*. were able to detect various proteins (involved in the skins regeneration process) in latent fingermarks placed on both polyvinylidene fluoride (PVDF) and on white paper [38]. The general technique used in that research was based on the Western blot approach involving blocking, incubation and numerous wash steps, resulting in a total contact time of around three hours in various aqueous-based solutions [299]. In a similar approach, Reinholz successfully detected and developed latent fingermarks by targeting albumin [128]. Fingermarks placed on porous surfaces including nitrocellulose and white paper were clearly visible even after being in contact with aqueous solutions for over three and half hours.

As was mentioned in Section 4.3.1, both of the lysozyme aptamers were selected to hen egg-white lysozyme and not human lysozyme. Although they had been shown to be successful in the detection of human lysozyme in other research, it was necessary to assess the specificity of the aptamers in this approach. In order to do this, both aptamers were tested against human and hen egg-white lysozyme at various lysozyme concentrations. Human and hen egg-white lysozyme were dissolved in buffer (see Section 4.4.3) to create lysozyme standards of 1 µg/µL, 0.1 µg/µL, and 0.01 µg/µL. These standards were pipetted (10 µL) onto PVDF and left to dry for 24 hours. The samples were incubated separately for up to three hours with either

of the aptamer sequences before being visualised. Results from these trials showed that there was no discernible difference between human lysozyme and hen egg-white lysozyme, with both aptamers being able to show clearly defined, fluorescent spots where the lysozyme standards had been pipetted (Figure 4-5 and Figure 4-6). With these results, further investigations into lysozyme affinity and fingermark detection could be undertaken; this is described in Section 4.4.

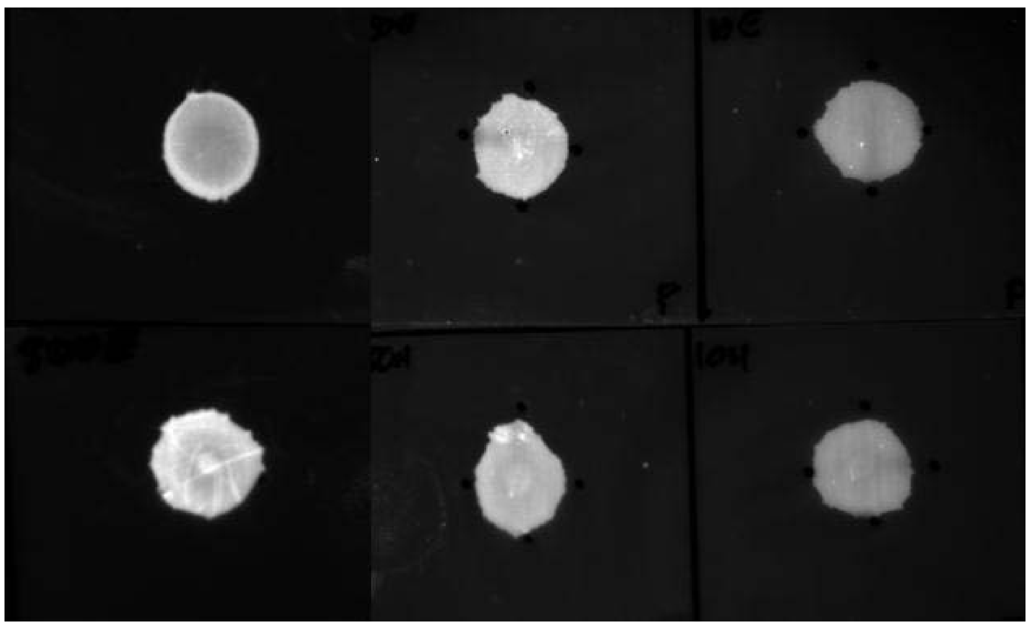


Figure 4-5: Lysozyme standards developed with aptamer 1 after incubation for 3 hour. Human lysozyme top, hen egg-white lysozyme bottom (10, 1, 0.1 µg left to right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

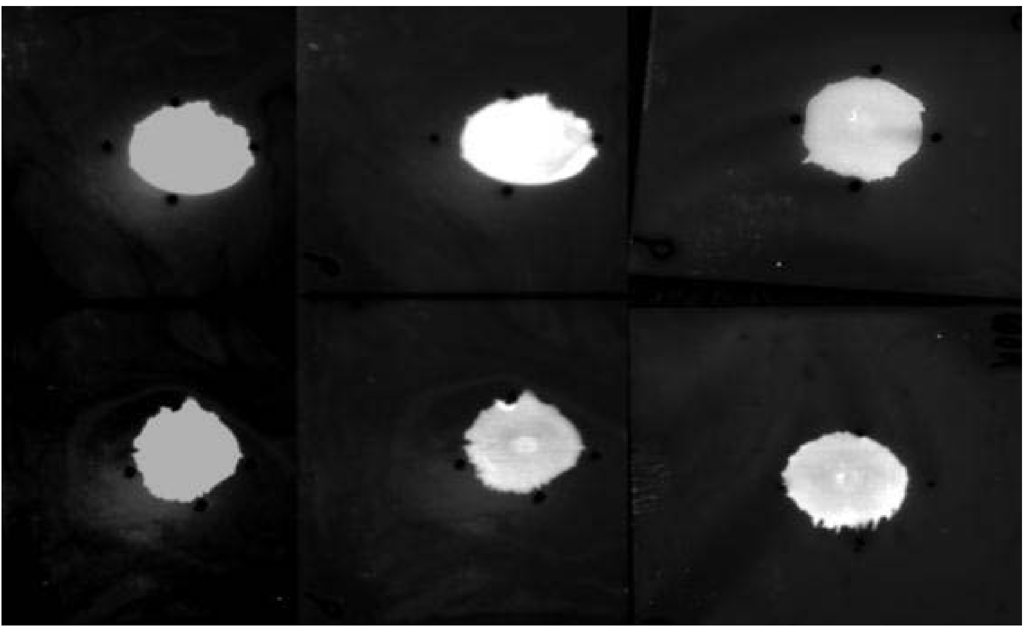


Figure 4-6: Lysozyme standards developed with aptamer 2 after incubation for 3 hours. Human lysozyme top, hen egg-white lysozyme bottom (10, 1, 0.1 µg left to right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

4.3.3 Selection of suitable surfaces

Although the main aim of this research was to detect latent fingermarks on commonly encountered porous surfaces such as paper, it was felt that with the earlier results (Chapter 3) and from the successful research of Drapel *et al*. and Reinholz, substrates more commonly used in protein detection would be the most suitable option for initial investigations (i.e. initial proof of concept). Substrates such as nitrocellulose and PVDF are regularly used in protein detection techniques such as Western, Northern and Southern blots due to their high protein capture and retention properties. Both substrates are highly non-reactive, therefore allowing the detection strategy to interact completely with the protein target. Both of these substrates were successfully used by Drapel *et al*. and Reinholz when using antibodies to detect proteins [38, 128].

Initially, both substrates were treated the same, with lysozyme standards being pipetted onto the substrate before being left for at least 24 hours. After this, both substrates were blocked using Western Blocker™ solution, an off-the-shelf blocking solution. However, when the substrates were visualised after incubation with either of the aptamers, the complete surface of the substrate was fluorescent (Figure 4-7 and Figure 4-8). With further investigation, it was found that the Western Blocker™ solution was the reason for the complete fluorescence

(Figure 4-9). Although the precise content of the Western Blocker™ solution is proprietary information, through personal communications it was found that lysozyme was present in the solution.

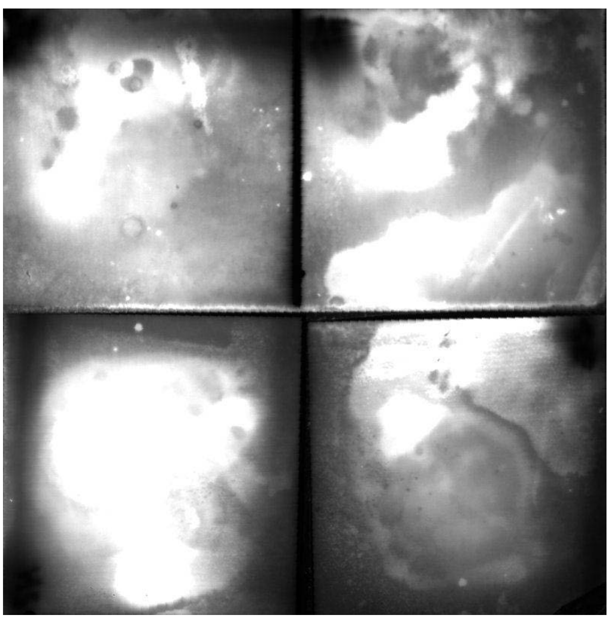


Figure 4-7: PVDF substrate after blocking with Western Blocker™ solution and subsequent development with aptamer 1 after incubation for 1 hour. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

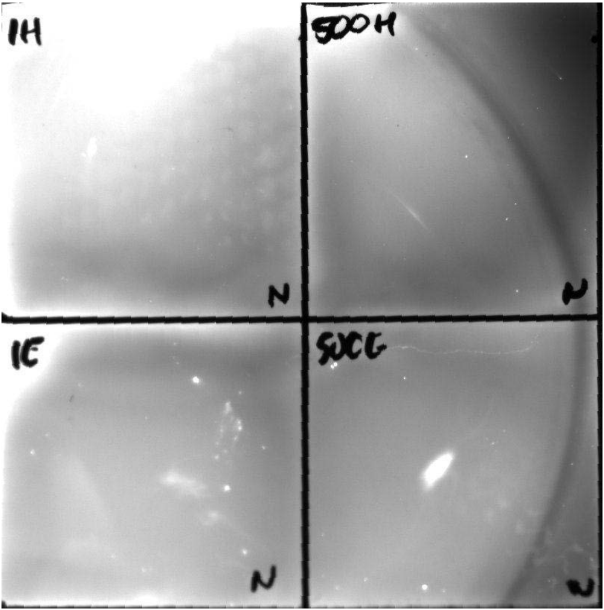


Figure 4-8: Nitrocellulose membrane after blocking with Western Blocker™ solution and subsequent development with aptamer 1 after incubation for 1 hour. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

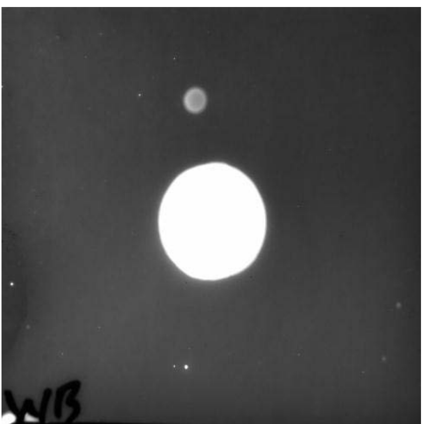


Figure 4-9: Western Blocker™ solution pipetted onto PVDF and developed with aptamer 2 after incubation for 2 hours. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

Before looking for a more suitable blocking solution, it was found that the PVDF membrane can be used without blocking provided that the membrane is thoroughly dried once the protein is added [300]. When the PVDF substrate was used in this way, it was found that clear lysozyme detection could be achieved without any non-specific interaction with the background (Figure 4-10).

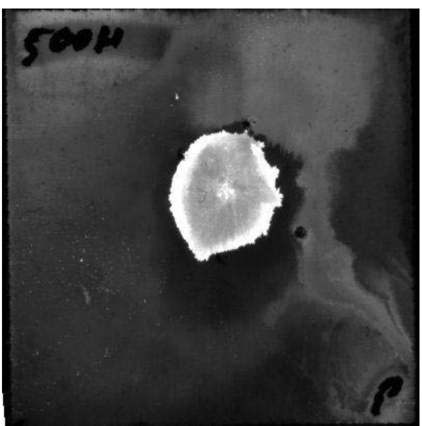


Figure 4-10: Human lysozyme (5 µg) pipetted onto PVDF without any blocking method. Lysozyme developed with aptamer 1 after incubation for 1 hour. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

When nitrocellulose was used in the same manner, however, no lysozyme detection was achieved. After the nitrocellulose substrates were visualised, only dark spots could be seen where the lysozyme had been placed initially; no aptamer interaction was present (Figure 4-11). The reasons for why this occurred are not fully understood; however, it may be due to

the pore size of the nitrocellulose membrane. The membrane used in this research had a pore size of 0.45 µm, which is suitable for larger protein targets (above 20 kDa) [301]. Unfortunately, membranes with a pore size of 0.2 µm (suitable for lower molecular weight proteins) were unavailable at the time. These would have been better suited for lysozyme as the molecular weight of lysozyme is 14.5 kDa.

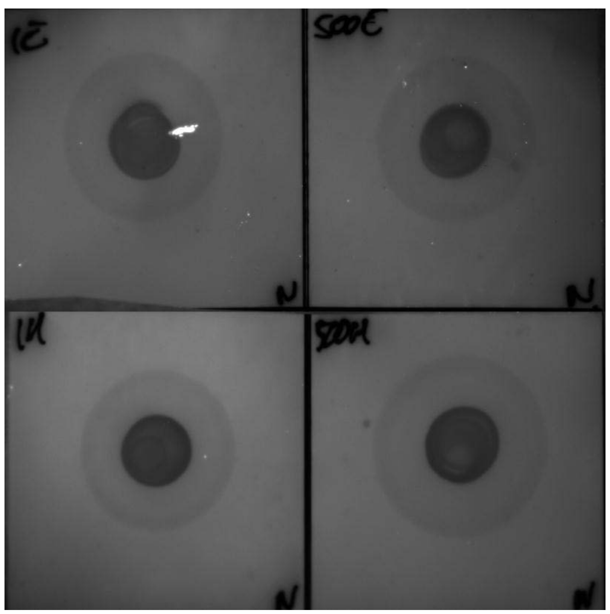


Figure 4-11: Nitrocellulose pipetted with hen egg-white lysozyme (top row) and human lysozyme (bottom row) at 10 µg (left) and 5 µg (right) after being in aptamer 1 solution for 1 hour. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

The issue with the non-retention of lysozyme was further displayed when the nitrocellulose substrate was tested with limited oligo buffer. Nitrocellulose substrates were pipetted with human and hen egg-white lysozyme at 10, 5, 2 and 1 µg amounts. By using the spray technique as used in Section 3.5.2, the nitrocellulose substrates were sprayed with 60 µL of aptamer 1 solution at 1 µM. Once sprayed, the substrates were imaged and recorded using the Poliview system under standard conditions. When imaged, clearly defined and highly fluorescent spots could be seen where the lysozyme had been placed. Fluorescent rings could also be clearly seen where the lysozyme solvent front had migrated to. These results confirmed binding of the aptamer to the lysozyme standards. However, when the developed substrates were placed in TBS buffer as a wash stage to remove excess aptamer reagent, almost all fluorescence was removed in the process (Figure 4-12). This illustrated the fact that, although lysozyme binding does occur on nitrocellulose, the nitrocellulose substrate is unable to retain the lysozyme when in contact with an aqueous solution.

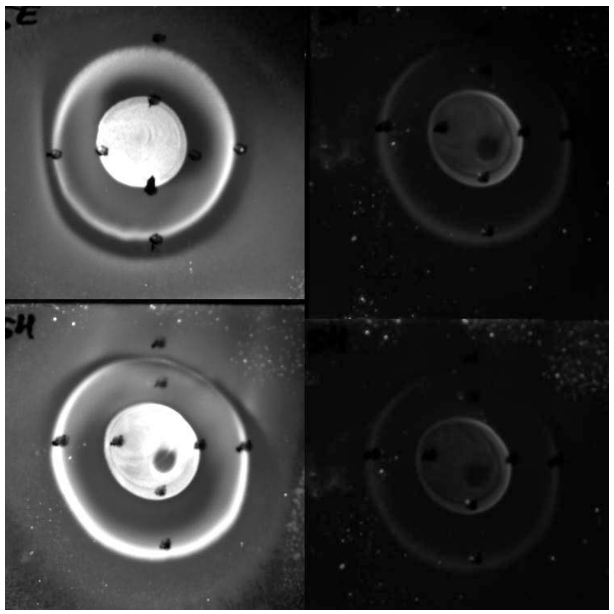


Figure 4-12: 10 µg lysozyme standards placed on nitrocellulose and developed with aptamer 2 using the spray technique (left) and developed lysozyme standards after TBS wash for 30 minutes (right). Hen egg-white lysozyme (top row) and human lysozyme (bottom row). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

4.3.4 Collection of latent fingermarks and storage

The fingermark donors used in this part of the research were the same as had been used previously. By using the same donors this meant that the general type of fingermarks deposited by each donor was already known.

Due to the limited availability of PVDF substrates, the size of the surface used for testing was generally kept quite small. This therefore meant that only one or two fingers could be placed onto the test sample. Donors were asked to place their middle finger and, if possible, their forefinger onto the substrates with gentle pressure. Both natural and charged marks were obtained from all donors (see Section 3.3.5). Marks were generally stored in the light in clear glass trays with a loose fitting cover. The temperature range of the laboratory where the samples were stored ranged from 21–28 ^{O}C with relative humidity between 30–55%.

For the comparison of different storage conditions, some fingermark samples were stored at room temperature while others were stored at 4 ^{O}C and at -20 ^{O}C using a laboratory fridge and freezer. Split marks were also used in sensitivity trials involving aptamer- and antibody-based reagents. In this work, donors were asked to place three fingers onto PVDF with gentle

pressure. After, the PVDF was cut vertically down the centre, allowing for the same fingermark to be developed with both reagents (Figure 4-13).

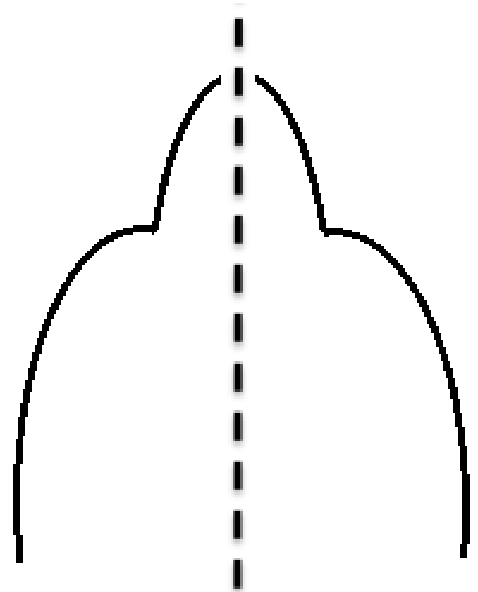


Figure 4-13: Representation of split marks used in the comparison studies.

4.4 Materials and methods

4.4.1 General

4.4.1.1 Reagents

DNA aptamers selected to hen egg-white were synthesised by Biosearch Technologies Inc., USA. Fluorescent tag modifications were undertaken during synthesis. Aptamers were received lyophilised and used as supplied.

Hen egg-white lysozyme [CAS 12650-88-3] dialyzed, lyophilized powder ≈100000 U/mg; human lysozyme [CAS 12671-19-1] recombinant, expressed in rice ≥100,000 units/mg; Trizma® base [CAS 77-86-1] ≥99.9% (titration); potassium chloride [CAS 7447-40-7]; BioXtra, ≥99%, Western Blocker™ solution; Triton™ X-100 [CAS 9002-93-1] laboratory grade; bovine serum albumin [CAS 9048-46-8] BioReagent; gelatin from porcine skin [CAS 9000-70-8]; squalene [CAS 111-02-4] (≥98% liquid); oleic acid (≥99% GC) and palmitic acid (≥99%) were obtained from Sigma-Aldrich and used as supplied.

Fluorescent Orange 550 reactive (≥90%) was obtained from Sigma-Aldrich and reconstituted in 50 µL amine-free anhydrous dimethylformamide prior to use.

D-Glucose [CAS 921-60-8] (anhydrous, UNIVAR) was obtained from Ajax chemicals and used as supplied.

Ethylenediaminetetraacetic acid [CAS 60-00-4] (anhydrous, ≥99%) was obtained from APS chemicals and used as supplied.

Sodium chloride [CAS 7647-14-5] was obtained from Chem-Supply and used as supplied.

Polyclonal anti-human lysozyme produced in rabbit was supplied by Lifespan Biosciences Inc, USA. Antibodies were received lyophilised from PBS and used as supplied.

Hydrochloric acid [CAS 7647-01-0] (36% w/v) was obtained from Univar Ajax Finechem and used to make 1 M HCl solution.

Ethanol [CAS 64-17-5] was obtained from AnalaR NORMAPUR® VWF and used as supplied.

4.4.1.2 Instrumental

Whatman protran nitrocellulose membranes (0.45 µm, 15 cm x 15 cm) and Immobilon®-P polyvinylidene difluoride (9 cm x 12 cm) were obtained from Sigma-Aldrich and used as supplied.

A Rofin Poliview IV imaging system with a Rofin PL500 Polilight forensic light source was used for the visualisation of treated lysozyme spot tests and fingermarks.

A Ratex instruments vortex mixer was used for the mixing of oligo solutions.

A Kelvinator opal fridge/freezer was used for the storage of DNA aptamers and solutions.

A Paton scientific encounter orbital shaker was used for the agitation of aptamer solutions.

An Eppendorf 5702 centrifuge was used for the spinning down of the oligos.

Aptaca 90 mm sterile polystyrene petri dishes were obtained from Livingstone International.

Gladfoil heavy duty aluminium foil was used to cover the development solution.

Amicon® Ultra 0.5 50K cellulose membrane centrifugal filter units were obtained from Merck Millipore and used as instructed to purify dye-tagged antibodies prior to use.

An Eppendorf minispin plus microcentrifuge was used for Amicon spin column purification for dye-tagged antibodies.

4.4.2 Development of aptamer-based reagent

Both sequences were re-hydrated using tris-buffered saline (TBS) buffer (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 8) to create stock solutions of 0.1 mM. Once TBS was added, the tubes were vortexed for one minute before being spun down via centrifuge for 30 seconds. For use in the developing solution and ease of storage, the stock solutions were further diluted using TBS to a concentration of 0.01 mM. To create the developing solution, 30 mL of TBS was added to a sterile petri dish. A 200 µL aliquot of either aptamer solution was then added and stirred. At all times the tubes and petri dishes were protected from light by using aluminium foil. All solutions were stored in the dark at 4 ^{O}C when not being used.

4.4.3 Application of reagent to lysozyme and fingermarks

Human and hen egg-white lysozyme standards were prepared by dissolving either lysozyme in a buffer. The buffer was made by adding 100 mM Tris, 2 mM ethylenediaminetetraacetic acid (EDTA) and 0.05% Triton X100 to deionised water. The pH was adjusted to pH 8 using 1 M HCl. 10-fold dilutions of lysozyme were prepared from the stock solutions to provide various lysozyme standards ranging from 1 µg/µL to 0.1 ng/µL. A 10 µL aliquot of each lysozyme standard was pipetted onto the PVDF substrate and left to dry for 24 hours at room temperature. The substrates were kept away from contamination during drying by placing them in a clean glass dish and covering loosely. Fingermark samples were collected and stored as detailed in Section 4.3.4.

Lysozyme and fingermark samples were placed into the developing solutions (see Section 4.4.2) with the fingermark side face down. The petri dishes were placed onto an orbital

shaker set at 40 rpm and covered with foil (Figure 4-14). The samples were left to incubate while being constantly agitated for between one to four hours at room temperature (approx. 18-22 ^{O}C). After this time, samples were visualised using the Poliview system with a 505 nm excitation source and 555 nm band-pass camera filter.

Figure 4-14: Incubation of PVDF samples with aptamer solution (in petri dishes) while being agitated with the use of an orbital shaker.

4.4.4 Identification of selectivity

The selectivity of the aptamers was investigated by testing the aptamer reagents against two commonly used proteins and four other large fingermark components. Glucose, palmitic acid, squalene and oleic acid, together with bovine serum albumin (BSA) and gelatine, were all used for testing. Glucose, BSA and gelatine were diluted in water to a concentration of 1 µg/µL, while oleic acid, palmitic acid and squalene were diluted in ethanol to the same concentration. A 10 µL aliquot of each target was pipetted onto PVDF membrane and left to dry for 24 hours at room temperature. These samples were then subjected to the same development process as stated in Section 4.4.3 before being visualised.

4.4.5 Development of anti-lysozyme reagent

Anti-lysozyme (human) antibodies were re-hydrated with TBS buffer to create a stock solution of 1 mg/mL. To this solution, 7 µL of Fluorescent Orange 550 reactive (in DMF) was added before being mixed and left to incubate for two hours at room temperature. The solution was then separated into two 500 µL aliquots and pipetted into the spin columns. These were capped and centrifuged at 14,100 rpm (14,000 g) for 10 minutes. After this, the clear filtrate at the bottom of the tubes was removed before the spin columns were inverted into clean tubes and centrifuged at 6,000 rpm (2,400 g) for two minutes. The fluorescently-tagged antibody was successfully recovered from the separation tubes and was reconstituted to 500 µL with TBS. To create a developing solution, the antibody solution was added at the same amount as the aptamer reagent to 30 mL of TBS contained within a petri dish. At all times, the tubes and petri dishes were protected from light using aluminium foil. When not in use, all solutions were stored in the dark at 4 ^{O}C.

4.5 Results and discussion

4.5.1 Performance of reagent on lysozyme and fingermarks

During the specificity investigations (see Section 4.4.3), various concentrations of both oligos used in the development solution were trialled, as shown in Table 4-4. For lysozyme tests, it was found that when using the higher concentrated development solutions visualisation was often impaired due to the intense fluorescence shown by the developed lysozyme spots. At the two lower concentrations however, fluorescence was reduced and clear detection of lysozyme could be easily achieved. When dealing with latent fingermarks on the other hand, the solutions at the higher concentrations were far better than the least concentrated solutions, which gave very weak visualisation. It was therefore decided to use the median (0.01 mM) development solutions as this gave good results for both lysozyme and latent fingermarks.

Table 4-4: Ability of various concentrations of aptamer used in the developing solution to develop lysozyme and latent fingermarks. (tick illustrates detection clearly visible, cross refers to development either overpowering or being weak).

Aptamer concentration	Human lysozyme detection	Fingermark detection
0.1 mM	✗	✓
0.01 mM	✓	✓
0.001 mM	✓	✗

To assess the ability of the aptamer development solutions to detect lysozyme, both human and hen-egg white lysozyme were trialled at decreasing amounts. As has already been shown (see Section 4.3.2), both human and hen egg-white lysozyme could be detected on PVDF at 100 ng with both aptamers. In further testing, it was found that human and egg-white lysozyme could still be very easily detected and visualised at 1 ng on PVDF. Even at this low amount, detection still gave strong fluorescence comparable to the detection of lysozyme at higher amounts (Figure 4-15 and Figure 4-16). With the very little difference observed in lysozyme detection at low and high amounts, it was felt that this verified the detection capabilities of the lysozyme aptamer-based reagent, therefore lower amounts were not further investigated. Also, as demonstrated by Drapel *et al.*, the total protein amount in eccrine and mixed fingermarks was found to be on average 133 µg and 384 µg, respectively [38], therefore highlighting the high sensitivity of this reagent.

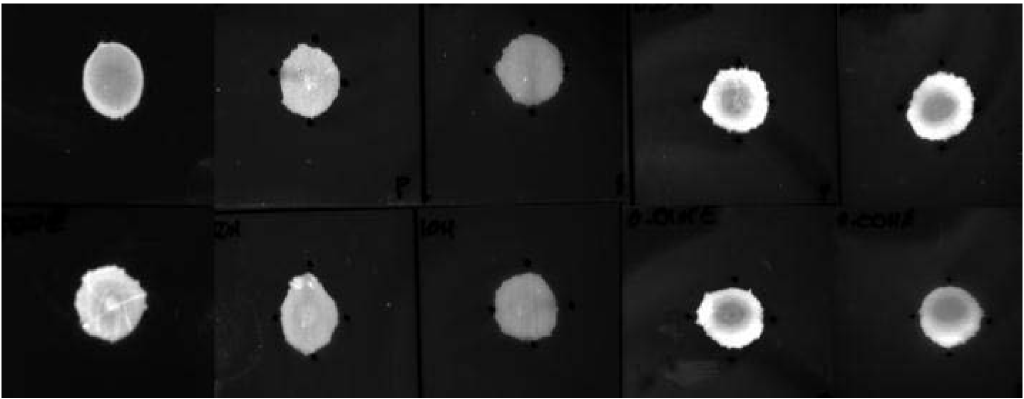


Figure 4-15: Lysozyme standards developed with aptamer 1 after incubation for 3 hours. Human lysozyme top, hen egg-white lysozyme bottom (10 µg, 1 µg, 100 ng, 10 ng, 1 ng left to right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

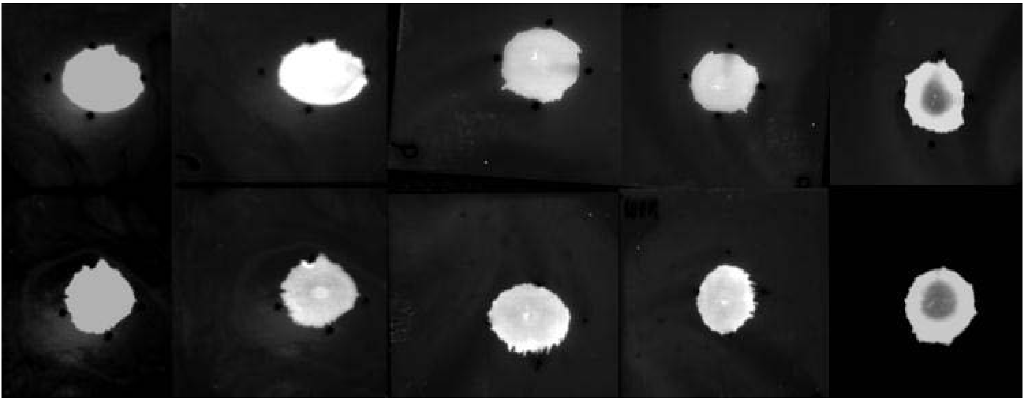


Figure 4-16: Lysozyme standards developed with aptamer 2 after incubation for 3 hours. Human lysozyme top, hen egg-white lysozyme bottom (10 µg, 1 µg, 100 ng, 10 ng, 1 ng left to right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

In initial fingermark trials, both natural and charged fingermarks were tested with both aptamer solutions. The first marks tested were those that had been placed on PVDF and developed on the same day. In these tests, it was found that natural marks produced no visible fluorescence, rather, dark marks with small black dots were visualised after any length of incubation. Charged marks, however, provided marks that exhibited fluorescence and clear ridge detail. Marks developed after an hour gave various results ranging from clear and strong development to weaker and less development but, after longer incubations (up to four hours), all marks were clearly visible with fluorescent ridges on a dark background (Figure 4-17 and Figure 4-18).

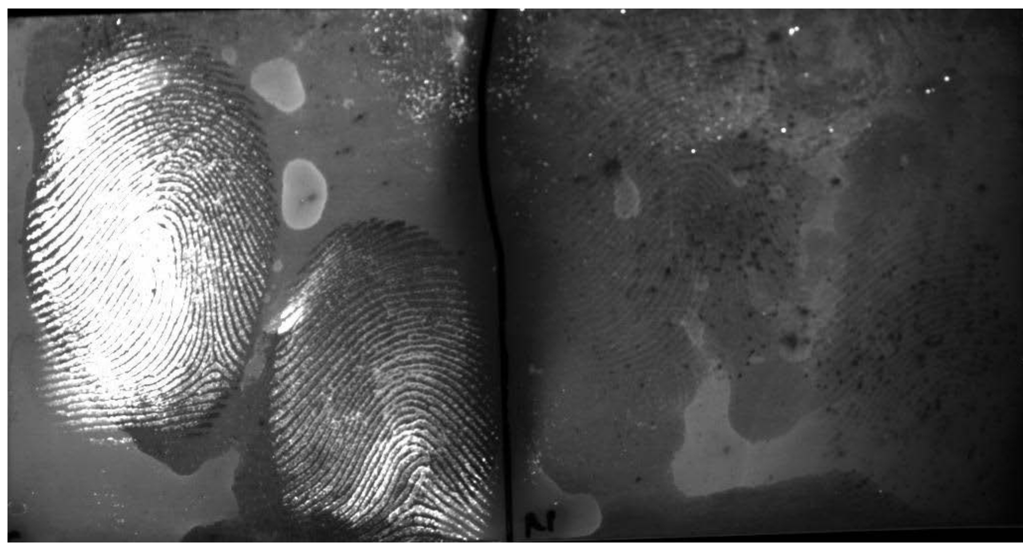


Figure 4-17: Fresh fingermarks developed using aptamer 1 after incubation for 4 hours, charged mark (left), natural mark (right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

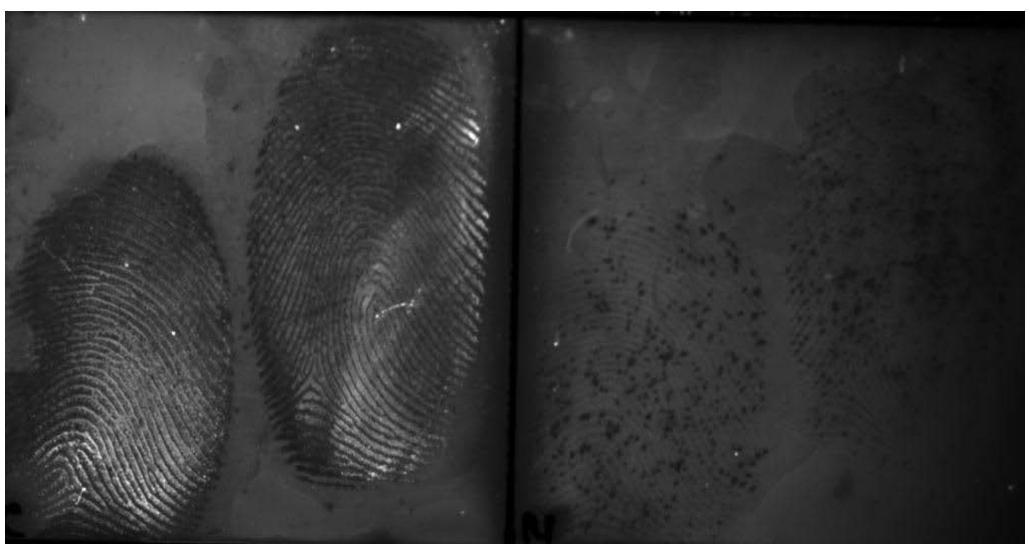


Figure 4-18: Fresh fingermarks developed using aptamer 2 after incubation for 1 hour, charged mark (left), natural mark (right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

As the aptamer-based reagents have already proven their highly sensitive ability to readily detect lysozyme down to 1 ng, the lack of results from natural fingermarks came as a surprise given that lysozyme is secreted by the eccrine sweat glands. These initial results with natural marks tend to show that either there is no lysozyme in natural fingermarks or that it is at too low a concentration for the aptamers to successfully interact. In order to investigate this

difference between charged and natural marks further, an alternative approach was sought to investigate the availability of lysozyme in natural fingermarks.

It was decided that the most suitable method to do this would be through a modified Western-blot approach very similar to the aptamer approach but with the use of antibodies targeted to human lysozyme. This approach was chosen as it is already a well-used and proven technique for the detection of many different proteins and hence would allow for a conclusive investigation into whether the aptamers were 'missing' the lysozyme on the PVDF membrane or that there was inadequate lysozyme present for aptamer interaction.

Polyclonal anti-human lysozyme antibodies were received from Lifespan Bioscience and prepared as described in Section 4.4.5. In order to allow for a fair comparison, the antibodies were tagged with a fluorescent tag with similar excitation and emission properties to that of the CAL-Fluor® Orange tag used with the aptamers. The dye chosen for this research was Fluorescent Orange 550 reactive, which allows for the attachment to the antibodies through amide coupling. The tagged anti-lysozyme antibodies were used to create a developing solution that was at the same concentration as with the aptamers. This developing solution was then placed into a petri dish ready for use. Before fingermarks were examined, the antibody based reagent was tested with lysozyme standards to ensure that the solution was working correctly. The antibody-based reagent was found to be able to detect human lysozyme down to the same quantity as the aptamer reagent, with 1 ng of lysozyme being easily visible when visualised using the Poliview (Figure 4-19).

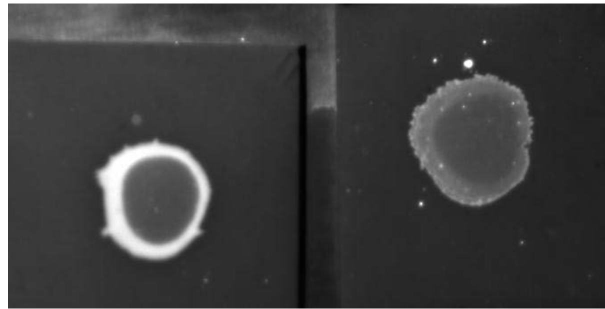


Figure 4-19: Lysozyme standards (1 µg, left, 1 ng, right) developed with anti-lysozyme development solution after incubation for 3 hours. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

With the successful detection of lysozyme, charged and natural fingermarks were then tested in the antibody-based reagent. Both charged and natural fingermarks were obtained from

three donors known to provide fingermarks that developed well with the aptamer reagents. These marks were cut vertically down the centre (see Section 4.3.4), allowing one side to be treated with the aptamer solution while the other side could be treated with the antibody reagent.

Results from these trials are shown in Figure 4-20 and Figure 4-21 and, as can be seen, there is very little difference between results achieved with the aptamer-based reagent and those from the antibody-based reagent. There is, however, a difference between results from the different donors. Two donors still did not produce any ridge detail with natural marks; however, the third donor did produce limited ridge detail. This difference is believed to be due to prior contamination of the donors fingers with sebum thus causing the donor's fingermarks to not be completely "natural" (uncharged). When the antibody-based reagent was used on the charged fingermarks, very similar results were achieved to those of the aptamer-based reagents, with very clear level 2 detail obtained.

It can be seen that the results obtained with the antibody clearly support the results achieved earlier with the aptamers. This shows that the aptamer-based reagents are interacting with the lysozyme within a charged latent fingermark. The main conclusion from the results with the antibody-based reagent is that natural fingermarks cannot be detected through the targeting of lysozyme. This would, therefore, appear to suggest that lysozyme is not present in adequate quantities in a purely natural fingermark. In comparison, the reason why charged marks are able to develop so well when targeting the same component is due to the loading of the fingermark with various components before the marks are placed. As the fingers are run over the donors forehead (an area known for high protein concentration [38]), lysozyme (together with other proteins) is loaded onto the finger, which in turn allows for successful transfer of the material onto the PVDF membrane. It is therefore believed that, in order to detect latent fingermarks through the targeting of lysozyme, the finger of the donor must be charged to allow for the successful transfer of lysozyme in the latent fingermark deposit.

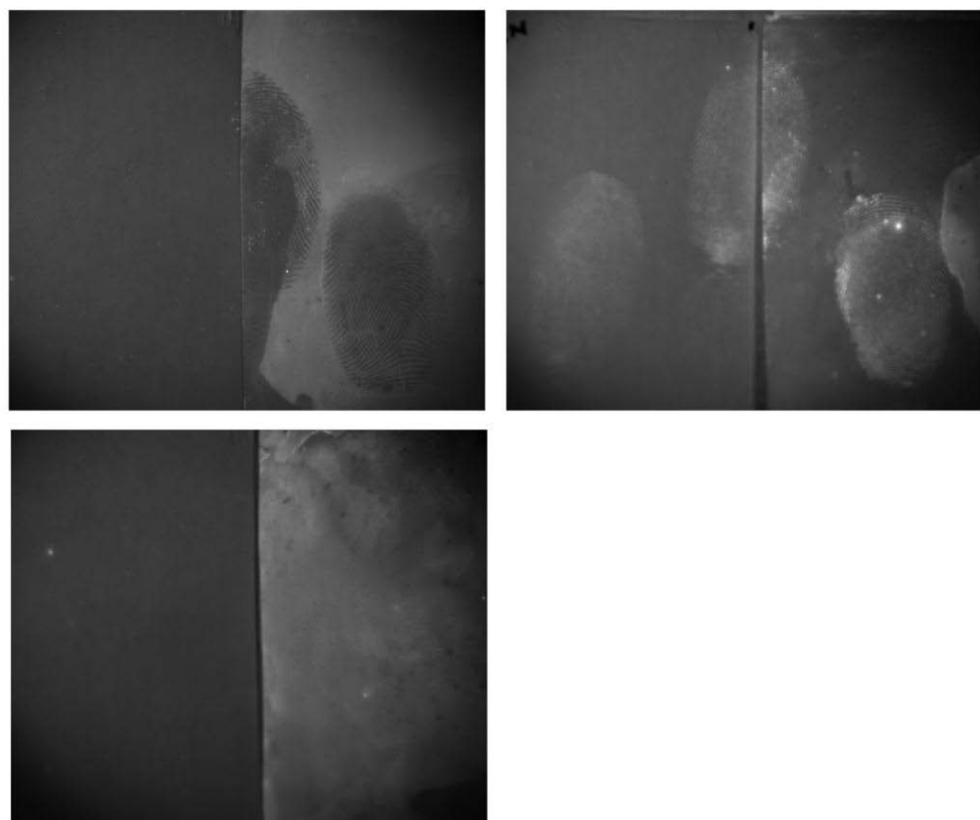


Figure 4-20: Fresh natural fingermarks placed by donors 1 (top left), 2 (top right) and 3 (bottom). Fingermarks developed with antibody-based reagent (left side) or aptamer 1 (right side) after incubation for 3 hours. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

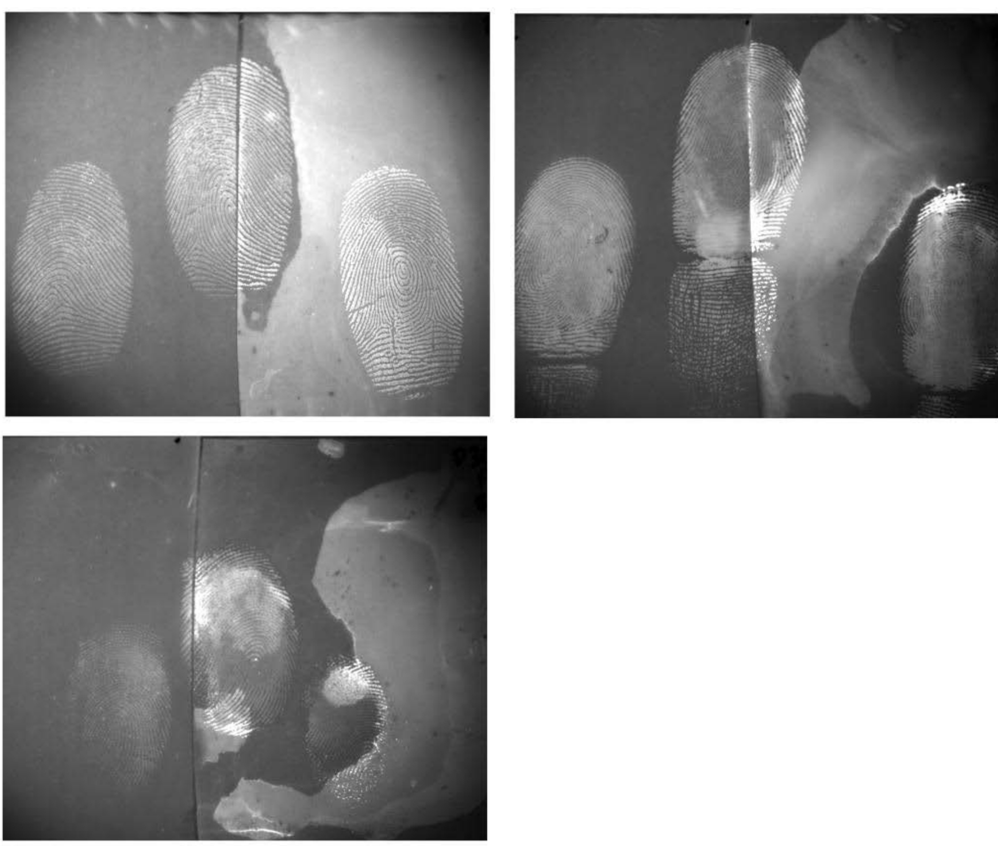


Figure 4-21: Fresh charged fingermarks placed by donors 1 (top left), 2 (top right) and 3 (bottom). Fingermarks developed with antibody-based reagent (left side) or aptamer 1 (right side) after incubation for 3 hours. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

Obviously, the issue of not being able to develop natural fingermarks is a major drawback with the aptamer-based reagents. This could easily be overcome by producing aptamers targeted to fingermark constituents that are in higher abundance, for example albumin. However, as has already been stated, this is something that was not possible in this project, with lysozyme being the only potential target and aptamer combination available. Therefore, as successful results have been achieved with charged fingermarks, the investigation into lysozyme and aptamers for the development of charged marks will be the main aim for the rest of this chapter, with less focus on natural fingermarks.

Although freshly placed charged fingermarks were able to be clearly detected using either of the aptamer reagents, aged marks were less consistent in the results obtained. Charged fingermarks, once placed, were aged for seven and 14 days before being processed in the exact same manner as with the fresh fingermarks. A fresh charged fingermark was also developed at the same time to ensure that the solution was working correctly. In the seven

day trial, two male donors were employed, both known to be strong eccrine and sebaceous donors (Donors 1 and 2). In the 14 day trial, the two male donors plus a female donor known to be a good eccrine donor were tested (Donor 3). In the seven day trial, both donor marks were very poorly developed. Donor 1 marks were not able to be developed at all, just producing strong dark marks, while donor 2 marks produced 'patchy' fingermark detail but only after a four hour incubation time (Figure 4-22). It was found that increasing incubation times further did not improve on the detail already developed.

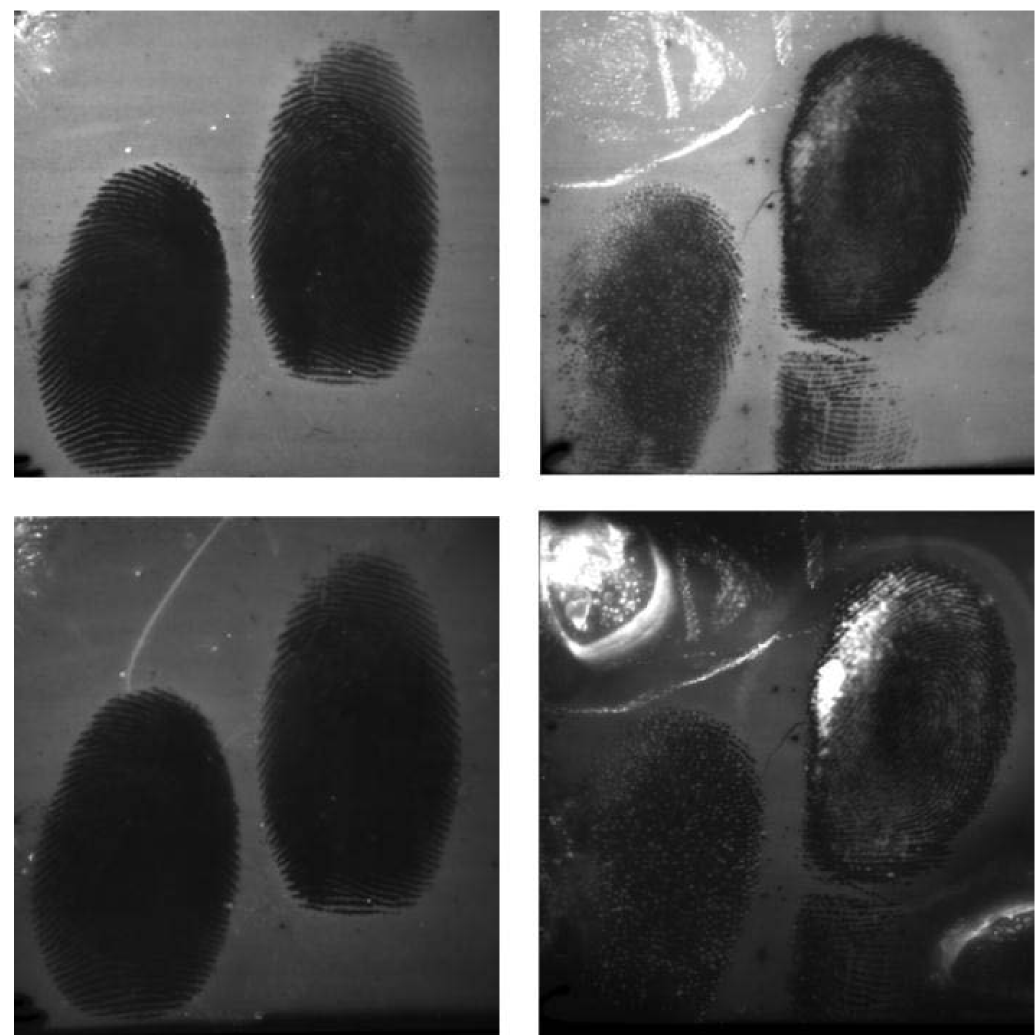


Figure 4-22: Aged donor fingermark (7 days) development using aptamer 1 after 1 hour (top) and 4 hour (bottom) incubations. Donor 1 (left), donor 2 (right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

While the fingermarks developed in the 14 day trial were also poorly developed, there was slightly greater detail seen than had been achieved in the seven day trial. Again, donor 1 produced almost no detail, with only small areas of the mark showing ridge detail after a four

hour incubation. Donor 2 produced improved results than before, with strong fluorescent detail visible, although again very 'patchy' marks were developed that obscured a large amount of the fingermark detail. Donor 3 produced no visible detail after one hour incubation but, after four hours, limited ridge detail was visible but this was generally in the form of fluorescent dots providing the ridge pattern (Figure 4-23 and Figure 4-24).

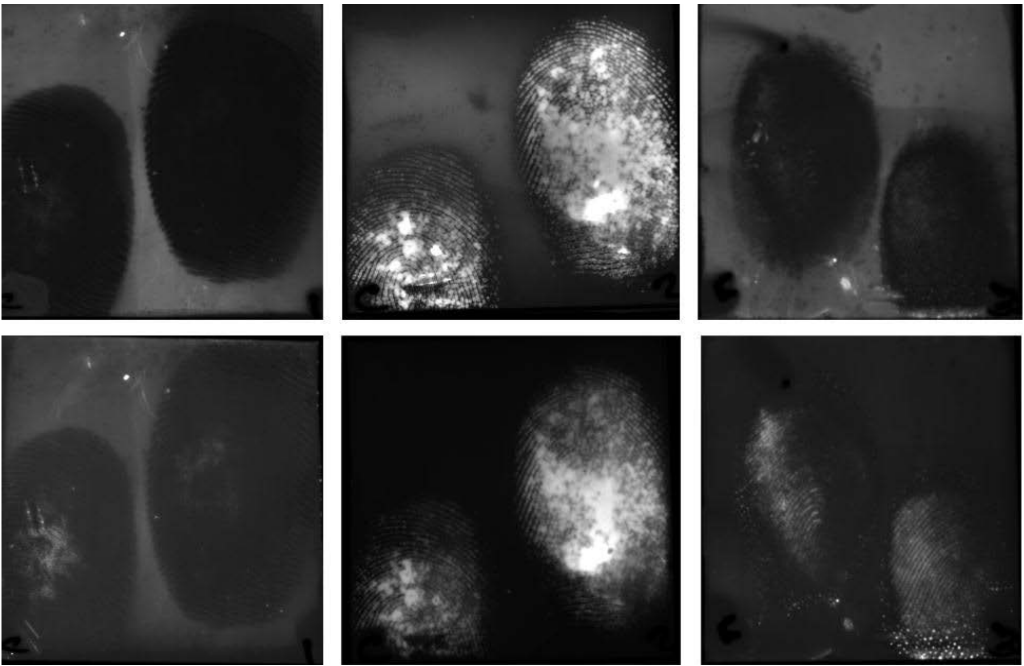


Figure 4-23: Aged donor fingermark (14 days) development using aptamer 1 after 1 hour (top) and 4 hour (bottom) incubations. Donor 1 (left), donor 2 (middle), donor 3 (right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

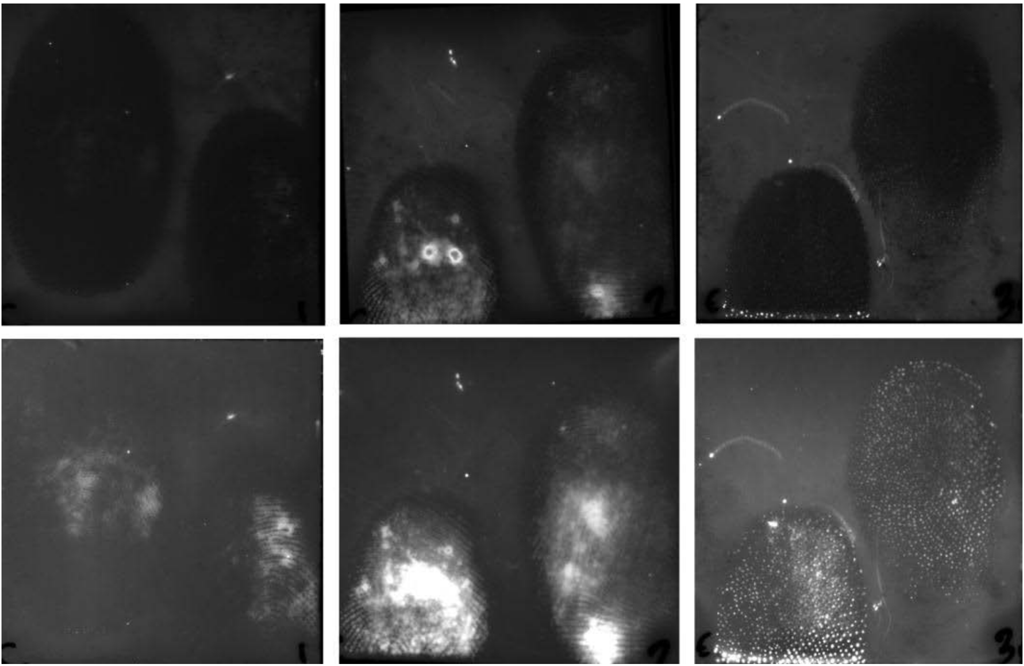


Figure 4-24: Aged donor fingermark (14 days) development using aptamer 2 after 1 hour (top) and 4 hour (bottom) incubations. Donor 1 (left), donor 2 (middle), donor 3 (right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

As has been shown in a number of research articles, the composition of latent fingermarks once deposited onto a surface can change quite dramatically in a relatively short period of time [26, 32, 232, 243, 302]. Also stated in much of this research is how the actual amounts of various fingermark components differ vastly from donor to donor when placed and aged. These donor differences can be due to factors including diet, gender, age and deposition conditions [233]. With this prior understanding of how latent fingermarks 'behave' once deposited, it is clear to see that the results obtained with these aged marks has followed this trend, with the composition of the marks becoming altered over time, causing the aptamer reagents to become less successful. Results also illustrate how differences in donor characteristics can dramatically affect development between donor marks.

Studies on the degradation of fingermark components over time have only really focussed on amino acids and the sebum constituents (fatty acids, wax esters, squalene). Proteins, unfortunately, have not been investigated, most likely due to their limited use in latent fingermark detection. Many proteins are known to be relatively stable when in a de-hydrated state and are able to resume protein function once re-hydrated [303, 304]. However, this is not always the case, with certain drying methods and the inclusion of additives often needed to help protect the protein structure when drying [305, 306]. Results by Balme *et al*. have

shown that, by adsorption onto dry inorganic substrates, lysozyme does not undergo strong conformational changes when water is removed [307]. Further, hard proteins (including lysozyme) have been found to remain stable on porous substrates and retain their structural properties when dried [308]. This would therefore tend to demonstrate that the structure of lysozyme will not alter by itself while retained dry on a porous substrate such as PVDF. This can be supported by the fact that, in initial trials with human lysozyme standards, detection could easily be achieved on lysozyme samples that had been left on PVDF for over six months.

With lysozyme being found to not degrade in its pure form when dried on PVDF, other factors must be interacting with the lysozyme to cause the poor results. The sebum composition of latent fingermarks could be the cause of the deterioration in fingermark quality through a number of possible interactions. The lipid component of latent fingermarks will often stay on the surface of, or, within the superficial layers of a porous surface, while the proteins will generally diffuse deeper into the porous matrix (depending on size). These varying rates and depths of migration could be one possibility for the issues encountered. While small organic molecules, such as indanedione, are partially soluble in organic solvents and are able to penetrate the lipid phase, the hydrophilicity of the aptamers and their size may limit their ability to penetrate a cured lipid layer, and thus, inhibit their ability to interact with the lysozyme. Another potential reason could be due to the mobility of the sebum components once the fingermarks were placed. As can be seen in some of the images (Figure 4-23 and Figure 4-24), marks all have blurring of fingermark detail to some extent and this mobility may affect the lysozyme deposition. One other possible reason could be the degradation of lysozyme by other fingermark components. It is well known that fingermarks can contain a wide variety of biological components including bacteria, enzymes and proteases [30, 233]. Although lysozyme is a bacteriolytic enzyme effective against many bacteria, it can be degraded through proteolysis by a number of proteases, with some being found to accelerate proteolysis when in the presence of long chain fatty acids [309, 310].

In order to investigate this further, marks from the same three donors were placed onto PVDF and stored under different conditions for seven, 14 and 30 days. Marks were stored either in daylight or dark at room temperature or at 4 ^{O}C or -20 ^{O}C. After storage, marks were subjected to the exact development procedure as used previously. Although, again, there was strong variance between donors, overall it could be seen that fingermarks that had been stored in the cooler conditions were able to provide fingermarks that contained much greater detail than those stored at room temperature (Figure 4-25).

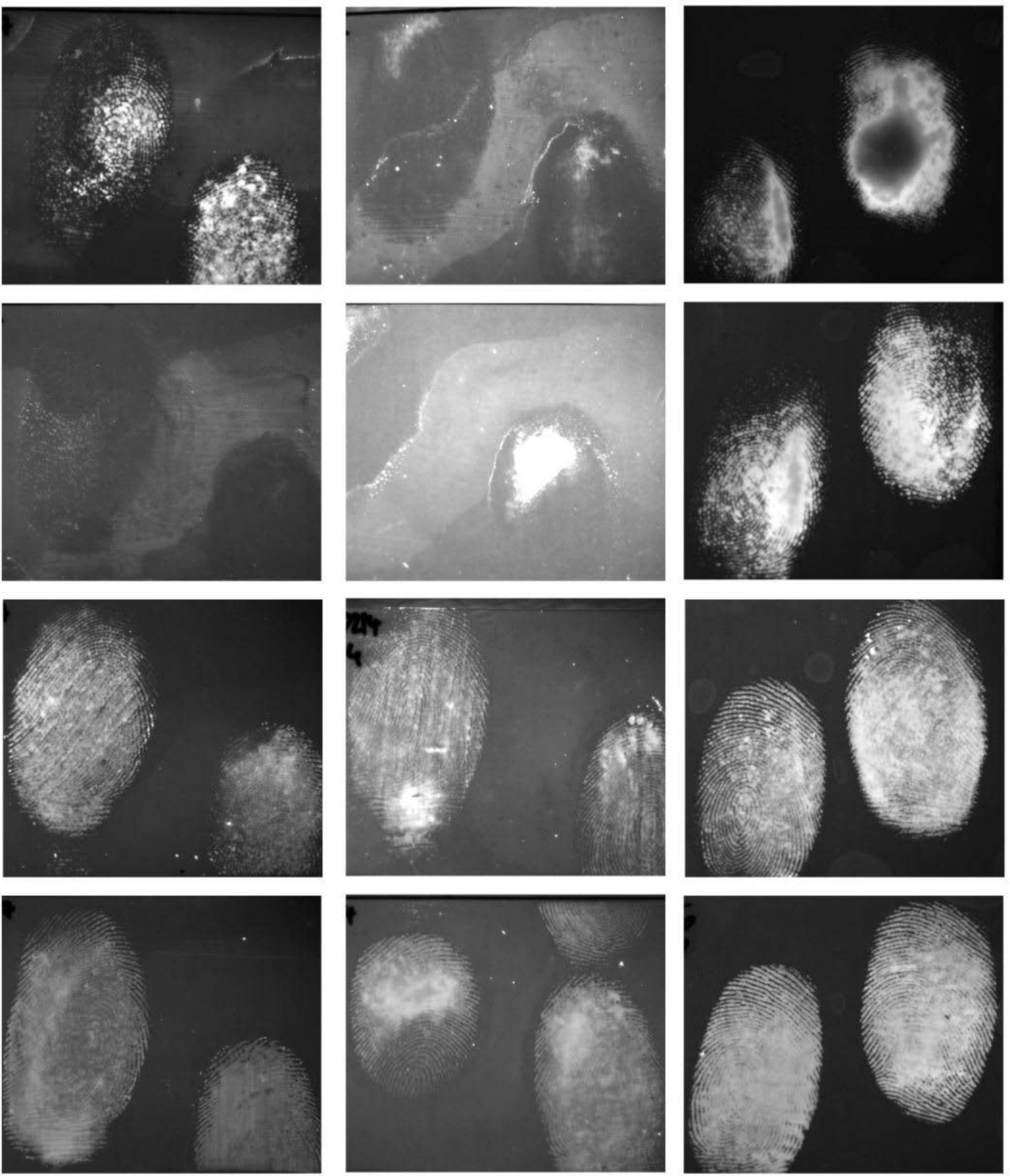


Figure 4-25: Results from the effect of storage conditions with charged fingermarks from donor 2 developed with aptamer 1 after incubation for 4 hours. Seven day aging (left column), 14 day aging (centre column) and 30 day aging (right column). Room temperature in light (top row), room temperature in the dark (second row), 4 ^{O}C (third row) and -20 ^{O}C (fourth row). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

As can be seen in the above figure (Figure 4-25), marks that have been stored at room temperature are the least well developed with very patchy and incomplete fingermark detail being observed. In comparison, those that have been stored in cooler and drier conditions show far more improved detail with clear ridge detail being visible in all of the marks. These results were found with all three donors, with -20 ^{O}C resulting in marks being developed with the best quality (other donor results are available in Appendix I).

These results tend to point to two main reasons as to why fingermark development is poor after the first couple of days post-fingermark placement, these being degradation and migration. As fingermark detail has remained clear and detailed in the chilled fingermarks but not in the room temperature marks, this suggests that lysozyme has been degraded possibly by bacteria, enzymes or proteases (as mentioned earlier). The majority of the marks developed with the greatest detail from all three donors were found to be with fingermarks stored at -20 ^{O}C. This temperature is often used to store biological items safely and prevent any degradation for short periods of time (< six months), while at 4 ^{O}C biological items are often safe from degradation for at least one month [311]. Migration of lysozyme may also be a reason for the poor development of latent fingermarks stored at room temperature. Although the relative humidity of the laboratory where the samples were stored generally never rose above 55%, the humidity at the cooler temperatures (in the fridge and freezer) is generally never above 10%. This difference in humidity may be enough to allow the lysozyme to migrate and hence cause the blurred marks that are seen with the room temperature stored impressions.

As was stated in Section 4.3.1, two different aptamer sequences to lysozyme were selected for use in this research. The two sequences selected were both DNA-based and had binding affinities in the low nanomolar ranges (31 nM aptamer 1, 2.1 nM aptamer 2). The only major difference between the two sequences was the length of the aptamer, with aptamer 1 being 30 bases long and aptamer 2 being 80 bases long. Although aptamers with longer random regions are more suitable in the selection cycle of aptamer generation, smaller and truncated aptamers have been found to be able to provide better aptamer interaction in certain circumstances [312-314]. Results seen in this work have shown no significant difference in the detection and development of both lysozyme spots and latent fingermarks on PVDF. Both of the aptamers have provided very similar results in all of the tests, ranging from clear and detailed latent marks being developed on fresh charged marks to blank and dark marks developed on certain aged marks (Figure 4-26).

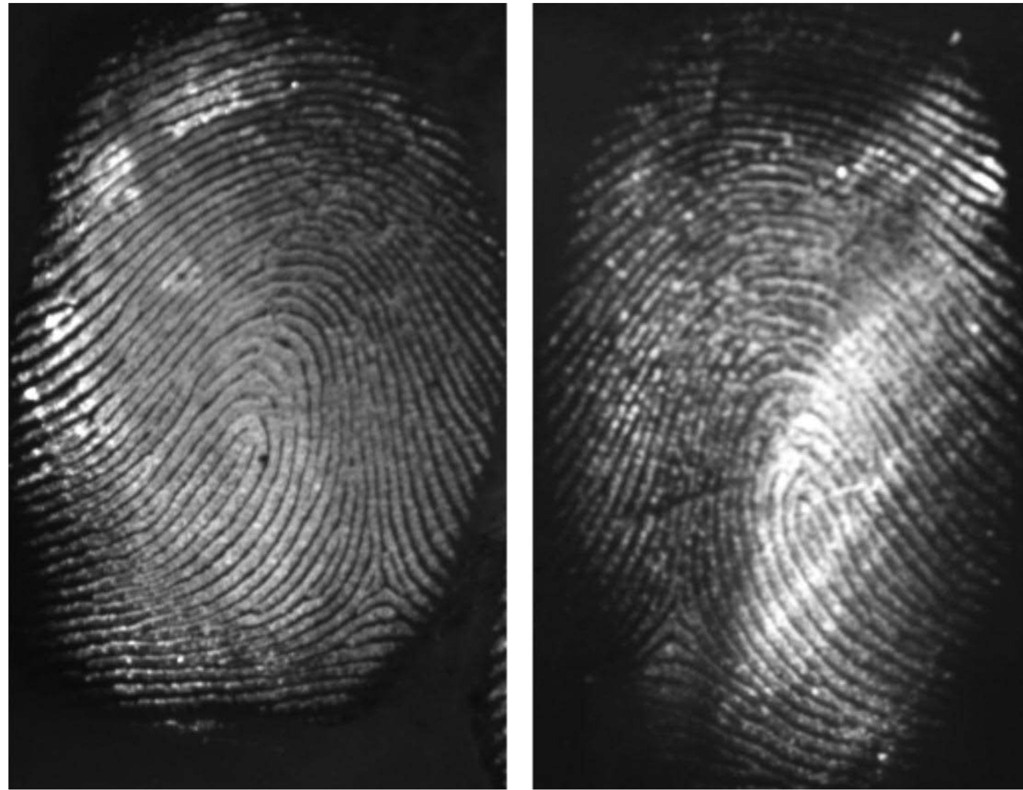


Figure 4-26: Charged latent fingermarks on PVDF, developed using the two aptamer-based reagents after incubation for 2 hours. The fingermarks were aged for 24 h before being developed and imaged under the same conditions, but with different lysozyme aptamers (aptamer 1 (left) and aptamer 2 (right)). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

One last thing to note in terms of the aptamer-based reagents is with regards to the shelf life of the prepared developing solutions. During the course of this research on PVDF membranes, all developing solutions were used until exhausted. Generally, developing solutions lasted only a couple of weeks until the aptamer concentration in the developing solutions became insufficient for fingermark detection. However, it was found that, even when the developing solutions had been left for long periods of time, they were still able to provide the same results as fresh solution. The longest period for storing development solutions (at 4 ^{O}C) was over five months, with the reagent still able to produce results as if it was freshly made (Figure 4-27). This highlights that these DNA-based aptamer reagents, if stored correctly, are very stable as fingermark development reagents.

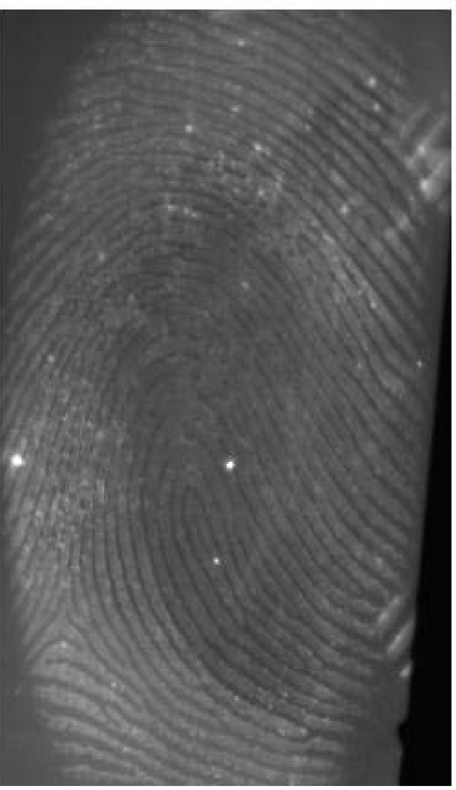


Figure 4-27: Charged fingermarks developed with aptamer 2 after incubation for 2 hours. Fresh solution (left) compared with a 5 month old solution (right). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

4.5.2 Suitability of Rofin Poliview for visualisation

The fluorescent tag chosen in this research, as detailed in Section 4.3.1, was CAL Fluor® Orange 560. This dye was chosen due its excitation and emission properties being very similar to those of the HEX dye used in the previous chapter. Although the HEX dye was found to be responsible for non-specific interactions and had to be replaced, it was found to be a very suitable dye for visualisation using the Rofin Poliview system.

As the CAL Fluor® Orange 560 dye was similar in fluorescent properties, it was found that the optimum visualisation properties for the aptamer reagents were the same. A band-pass filter of 505 nm with a 40 nm band width was used for excitation, while a camera barrier filter of 555 nm with a 10-20 nm band width was used for visualising the emission. These settings were found to be suitable throughout the study, providing clear and detailed images with very limited background fluorescence. Again, this work showed that the Rofin Poliview system was very suitable as an imaging system to use with these reagents.

4.5.3 Identification of non-specific binding

In order to investigate any non-specific interactions shown by the aptamers, experiments were carried out to test the selectivity of the aptamers to other fingermark components. Due to the issues found with the ELISA approach for investigating specificity in Chapter 3, an alternative method was sought for this investigation. As the modified Western blot using PVDF membrane had been shown to be successful in the detection and visualisation of the lysozyme standards and latent fingermarks, it was felt that this method would be the most suitable to investigate the specificity of the aptamer reagents.

With the positive results seen in Chapter 3 for the non-specific binding of aptamers to the fingermark components glucose, squalene, oleic acid and palmitic acid, these same four fingermark components were chosen again for testing against the lysozyme aptamers. As well as these four fingermark components, the aptamer reagents were tested against two common proteins BSA and gelatin. All six of the targets were tested on PVDF membrane in exactly the same procedure as for lysozyme and latent fingermarks. Each target was tested at an amount of 10 µg, while for reference a 1 ng human lysozyme standard was also tested.

Results from these tests showed no affinity of either aptamer-based reagent to any of the fingermark component standards (other than lysozyme) or for the two proteins. Results were the same, with each aptamer reagent producing PVDF membranes with no detectable fluorescence (Figure 4-28). Only the 1 ng human lysozyme target was clearly detected, illustrating that the aptamer solutions were working correctly.

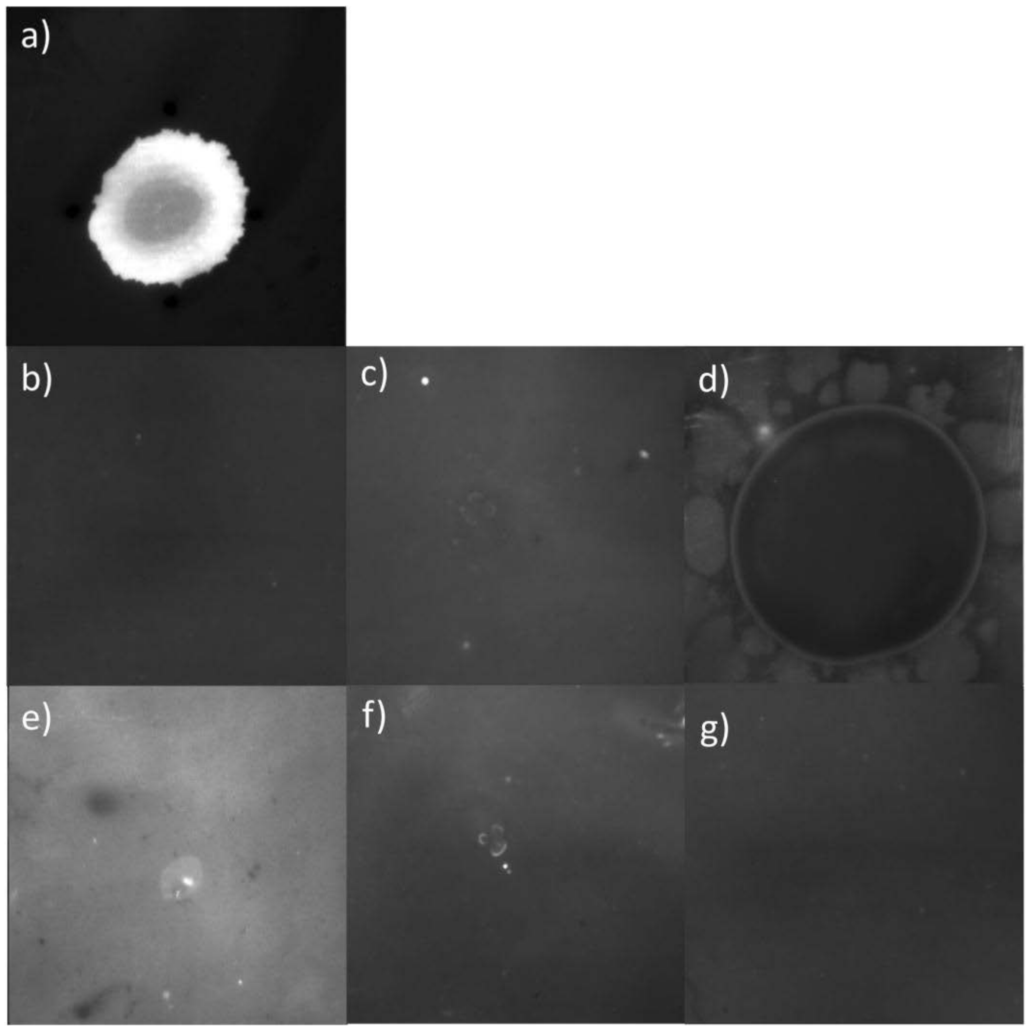


Figure 4-28: Demonstration of aptamer sensitivity and selectivity. The sensitivity of the lysozyme aptamer 2 reagent is demonstrated with the detection of 1 ng of lysozyme (a). Selectivity is demonstrated through the lack of binding with two proteins (BSA (b) and gelatine (c)) and a number of fingermark components (oleic acid (d), glucose (e), squalene (f) and palmitic acid (g)), all at 10 µg scale. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

Although it was found that the fluorescent tag used in Chapter 3 was responsible for the non-specific interaction, the fluorescent tag used in this research was not able to be tested due to it only being available through its attachment to an oligo. Although an investigation of the dye would fully confirm its non-participation in binding, it can be seen through both its chemical structure and the lack of affinity shown towards any of the other targets (shown above) that the successful detection of lysozyme is through the high sensitivity and selectivity of the aptamer-based reagents.

4.6 **Conclusions**

The research in this chapter set out to investigate the potential of DNA-based aptamer reagents for use in detecting and visualising latent fingermarks. With the knowledge gained from Chapter 3, the focus of this chapter moved away from the detection of small-molecular targets (amino acids) with RNA-based reagents and looked into the detection of larger targets. This allowed for the use of the more stable DNA-based aptamers while far greater binding affinities were possible, both of which proved to be issues in previous work (Chapter 3). The target chosen for this research was lysozyme, an enzyme known to be present on the skin's surface. Two published aptamer sequences specific for lysozyme were commercially synthesised and modified with the addition of a fluorescent tag. Again, with the issues encountered in Chapter 3, a different, tag was chosen that exhibited fewer non-specific interactions, but, still provided similar fluorescence properties.

In an approach to provide the aptamers and lysozyme with the optimum conditions for successful interaction, a modified Western-blot method was undertaken using PVDF as the membrane for lysozyme and fingermark investigations. Samples for testing were placed into an aptamer-based developing solution and left to incubate for up to four hours before being visualised using the Poliview system.

In initial results with lysozyme, it was found that both aptamer sequences were able to strongly detect and develop lysozyme down to 1 ng. Due to the use of PVDF membrane, no background interference was seen even with the long incubation time of four hours. When the aptamer-based reagents were tested on fingermarks, however, two very different results were seen with fingermarks that had been placed and developed within 48 hours. On natural fingermarks from all donors, very little and often no fingermark development could be seen. With charged marks, however, all donors produced strongly fluorescent fingermarks that had clear second-level features. To ensure that the lack of development with natural marks was not due to the aptamer-based reagents 'missing' lysozyme, an antibody reagent directed to lysozyme was also used. Results with this reagent also provided no development, therefore highlighting a major issue with natural fingermarks being that an insufficient quantity of lysozyme was present.

As well as the issue encountered with natural fingermarks, charged fingermarks that were aged for longer than three to four days also became problematic to develop. When marks

were aged, it was found that they became blurred, lost ridge detail and, in some cases, produced no visible impression. The reason for this has not been fully proven but the results indicate the role played by the degradation of lysozyme by other components present in the fingermark. When fingermarks were stored at low temperatures (temperatures used for the safe storage of biological items), fingermarks could be easily developed on marks that had been aged for up to 30 days, whereas those that had been left at room temperature for the same amount of time provided very poor to no detail.

Again, with the issues of non-specificity encountered in Chapter 3, the lysozyme aptamers were investigated for any non-specific interactions with other fingermark components. In this work, the clearly evident detection and visualisation of the lysozyme standard at the 1 ng level, and the absence of non-specific binding (for the other substances tested at much higher amounts), highlights the sensitivity and selectivity of the aptamer-based reagents. Although the fluorescent dye could not be tested, its stable structure points to purely aptamer-directed interactions with the lysozyme and fingermarks.

Overall, this chapter has successfully achieved the main aim of this project by providing the first proof of concept for the use of aptamers as a reagent for latent fingermark detection. However, this is not without many problems relating to fingermark composition. As has been previously discussed, these issues all stem from the use of lysozyme as the target for aptamer interaction. Unfortunately, this cannot be addressed in this project due to the limitations in aptamer selection; but, through the use of aptamers to other, more abundant and stable fingermark components, it is highly likely that the issues encountered here would not be seen and aptamer-based reagents would be far more successful.

Chapter 5: Detection of fingermarks on paper using the lysozyme aptamer-based reagent

Chapter 5: Detection of fingermarks on paper using the aptamer-based reagent

5.1 Introduction

The results achieved in Chapter 4 have, for the first time, demonstrated the potential of using aptamers for the detection and visualisation of latent fingermarks. Although this has provided the proof of concept sought in this project, the potential of aptamer-based reagents needs to be investigated further for use 'in the real world'. That is, in circumstances that are more commonly encountered by latent fingerprint investigators and technicians.

In Chapter 4, the general approach in detecting and visualising latent fingermarks with the use of aptamers was derived from one of the most commonly used immunological methods of detecting proteins, the Western blot. In this approach, in order to provide the best possible chance of detecting the protein of interest, a specific porous membrane with strong affinity for proteins is used. In this research, PVDF was found to be an excellent membrane on which to detect latent fingermarks through the targeting of the protein lysozyme. Unfortunately, PVDF is not a porous surface likely to be submitted for latent fingermark detection and examination.

Common porous surfaces often investigated for latent fingermark detection include paper, cardboard, some fabrics (e.g., cotton) and untreated wood [3]. Although very different in appearance and texture, all of these surface types are primarily composed of cellulose. As already stated (see Section 1.1.6), this allows amino acids in the fingermark deposit to be absorbed and become relatively stable within the porous surface, while a small amount of sebaceous content remains on the surface. PVDF, on the other hand, is a microporous thermoplastic fluoropolymer that binds proteins through hydrophobic interactions [315]. Although PVDF is chemically very different to the common porous surfaces mentioned, proteins detected in latent fingermarks on PVDF can also be detected on paper. Drapel *et al*. initially used PVDF to successfully develop latent fingermarks by targeting three different proteins involved in skin regeneration and protection [38]. Using the same technique, the three proteins were also detected in latent fingermarks placed on whitened and non-whitened paper, although these were more difficult to characterise compared to the marks

developed on PVDF [38]. Reinholz also showed that proteins detected in latent fingermarks placed on nitrocellulose (a common alternative to PVDF) could also be detected on a variety of paper types with similar results [128].

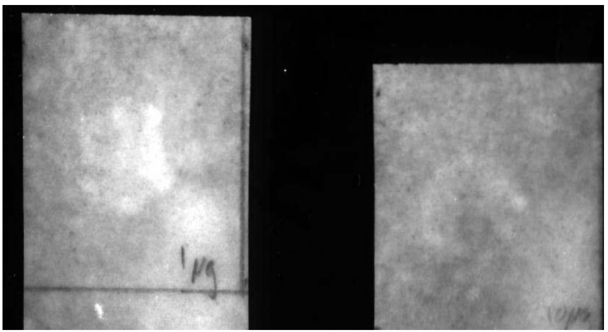
5.2 Objectives

In order to provide a more comprehensive analysis of the aptamer-based reagents developed in Chapter 4, the objective of this chapter was to investigate the potential of the reagents on more commonly encountered porous surfaces. The chosen surface for the research in this chapter was plain white copier paper, a surface that is both a common item of forensic evidence and also often used for the testing of new fingermark detection and visualisation reagents. The research in this chapter focussed on optimising the aptamer-based reagents for use on the paper surface, including protein fixation and surface washing techniques. In order to provide a definite answer to the potential of aptamer-based reagents, a comparison between marks developed by the aptamer method and those developed by IND-Zn was undertaken.

5.3 Experimental design

5.3.1 Reagent optimisation for use on paper surfaces

Using the optimised aptamer reagents, which had been successfully used in Chapter 4, on paper surfaces immediately caused development issues as can be seen in Figure 5-1. Initially, the reagents were used exactly as employed in Chapter 4 and were tested on lysozyme standards and charged fingermarks. It soon became apparent, however, that this approach was unsuitable for use on paper surfaces as very poor detection of the lysozyme standards was achieved, while charged fingermarks were unable to be developed using standard incubation times of one and four hours.



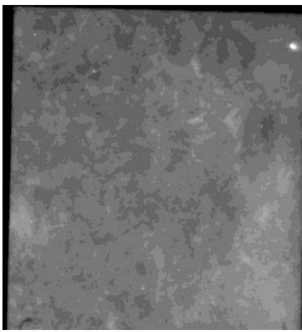


Figure 5-1: Lysozyme standards, 1 µg (left) 10 µg (middle) and fresh charged fingermark (right) developed with aptamer 1 after incubation for 1 hour. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

In order to improve on these results, both aptamer reagents were optimised for use on paper samples by investigating the following parameters:

- Aptamer concentration in solution
- Temperature increase of development solution
- Decrease in development time
- Buffer pH

Due to the previous results, it was believed that the incubation times were too long as it appeared that the lysozyme standards were being 'washed' off the surface causing poor detection and development. This is highly likely due to the vast difference in surface characteristics between PVDF and paper. Paper has a far greater porosity compared to PVDF; therefore, the protein can diffuse through the paper matrix to a greater extent due to less retention of the protein. This issue was also seen in the protein detection work on fingermarks by Drapel *et al*. [38]. With this in mind, shorter incubation times were investigated; these were five, 10, 20 and 30 minutes (Table 5-1).

Table 5-1: Result of charged fingermarks placed in aptamer 2 solution for increasing incubation times. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

Incubation time	Result
5 minutes	
10 minutes	
20 minutes	
30 minutes	

As seen in Table 5-1, the best development of charged fingermarks on paper was found to be between 20 to 30 mins. After 30 minutes, development decreased, eventually removing the earlier development. Although some development was possible, both detail and contrast is poor with only limited ridge detail being developed. Most of the mark, although fluorescent, is blurred in detail. This again highlighted the issue with protein diffusion in the paper. In order to try and overcome this diffusion issue, an increase in the aptamer concentration of the development solution was investigated. In the development solution used in Chapter 4, it was found that an aptamer concentration of 0.01 mM provided the best results (see Section 4.5.1). In this earlier work, it was found that a higher concentration of 0.1 mM was overpowering when used on human lysozyme due to high retention of lysozyme on the PVDF membrane. It was therefore decided that the 0.1 mM concentration may work well for use on paper due to the lack of retention and the high rate of diffusion of lysozyme.

As can be seen in Figure 5-2, the increase in aptamer concentration in the development solution produced a clear improvement in the detection and visualisation of charged fingermarks freshly placed on paper. Development times were also reduced to around 10 minutes depending on the level of development achieved. Although this provided an improvement over the earlier results, fingermark detail was still often blurred with ridge detail obscured or incomplete. One further drawback with the increase in aptamer concentration was that the background often became fluorescent. This is hypothesised to be due to an interaction between the DNA-phosphate backbone and the cellulose fibres (this is investigated in Section 5.5.1.2). In order to further improve the reagents and reduce these issues, an investigation was undertaken into the effect of development solution temperature.

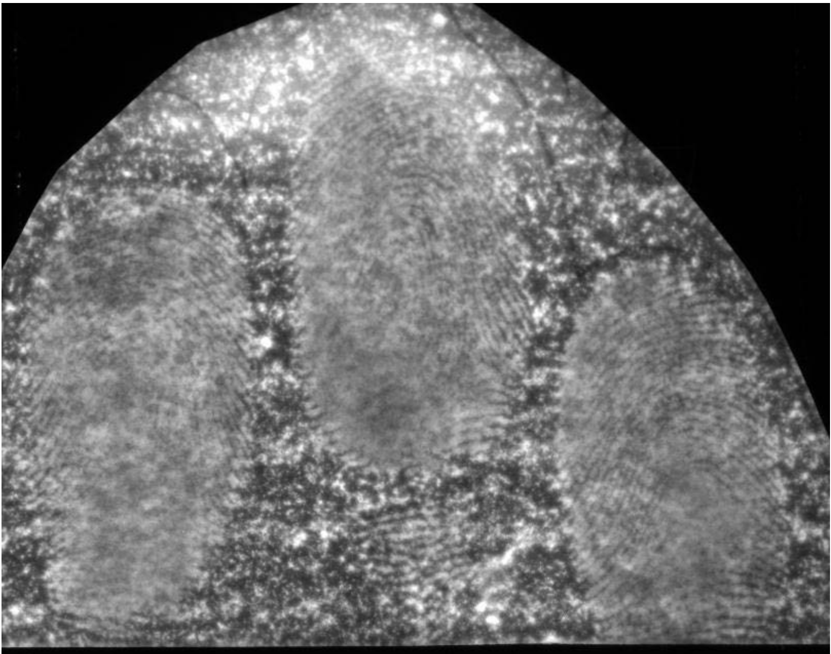
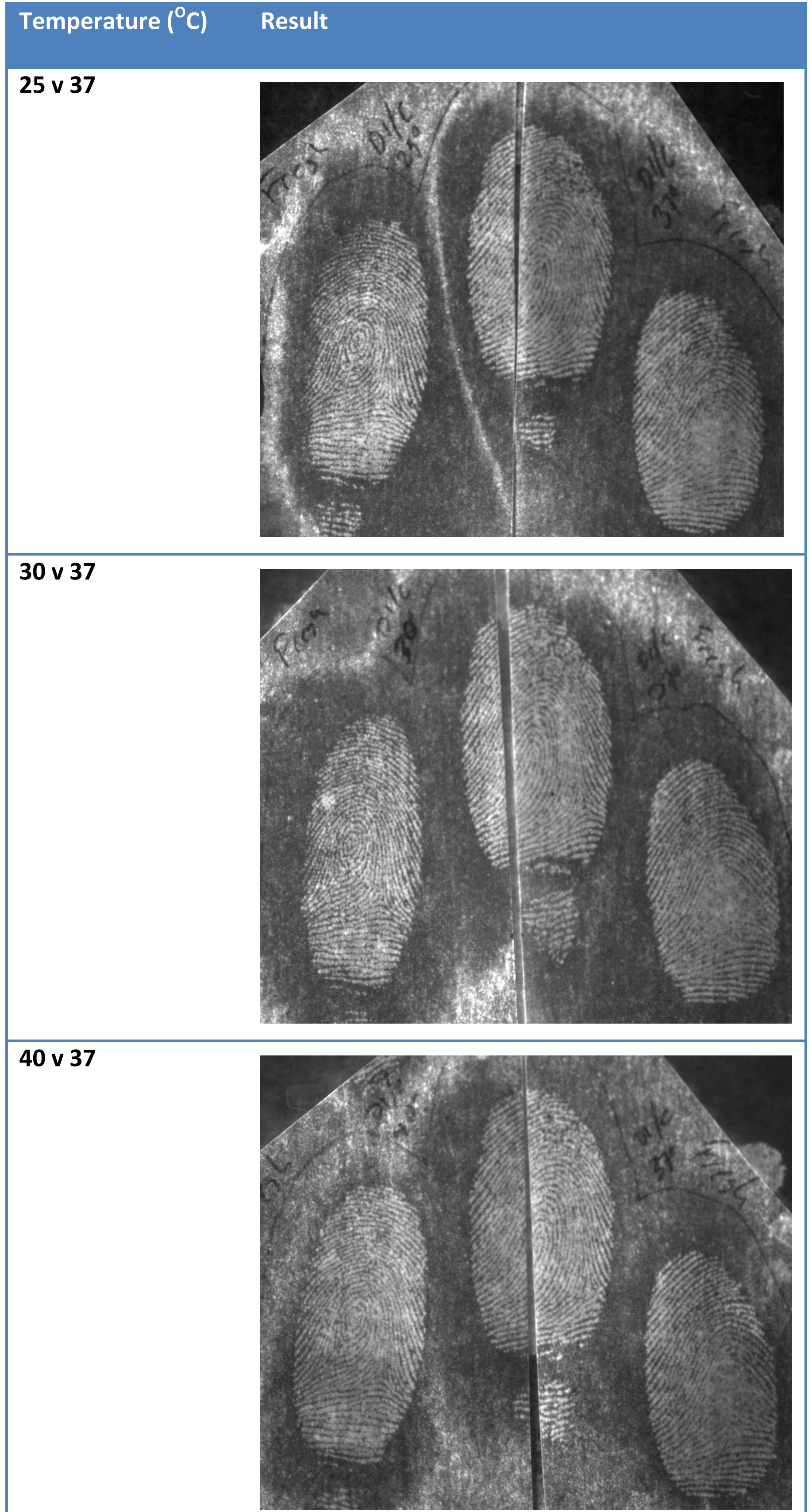


Figure 5-2: Charged fingermark freshly placed on paper and developed with an incubation time of 10 mins in aptamer 2 development solution with an increase in the aptamer concentration to 0.1 mM. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

Temperatures above normal room temperatures were investigated. It was felt that this may help to speed up aptamer interaction with the protein target and hence reduce the time required for incubation, thus, leading to less background interaction. Four temperatures were tested; these were 25, 30, 37 and 40 ^{O}C. Temperatures above this were not investigated as it may lead to the degradation of the aptamers and the fluorescent tag. Results from these trials showed that, by increasing the temperature of the development solution above 25 ^{O}C, clear ridge detail could be seen without the issues encountered previously (Table 5-2). With the increase in development temperature, it was found that incubation time was reduced dramatically. Instead of requiring 10 minutes to produce suitable development, an incubation time between 10 and 20 seconds was sufficient for optimum development.

Table 5-2: Results from charged fingermarks freshly placed on paper and developed with aptamer 1 at 0.1 mM with an incubation of 10 seconds and held at different solution temperatures. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

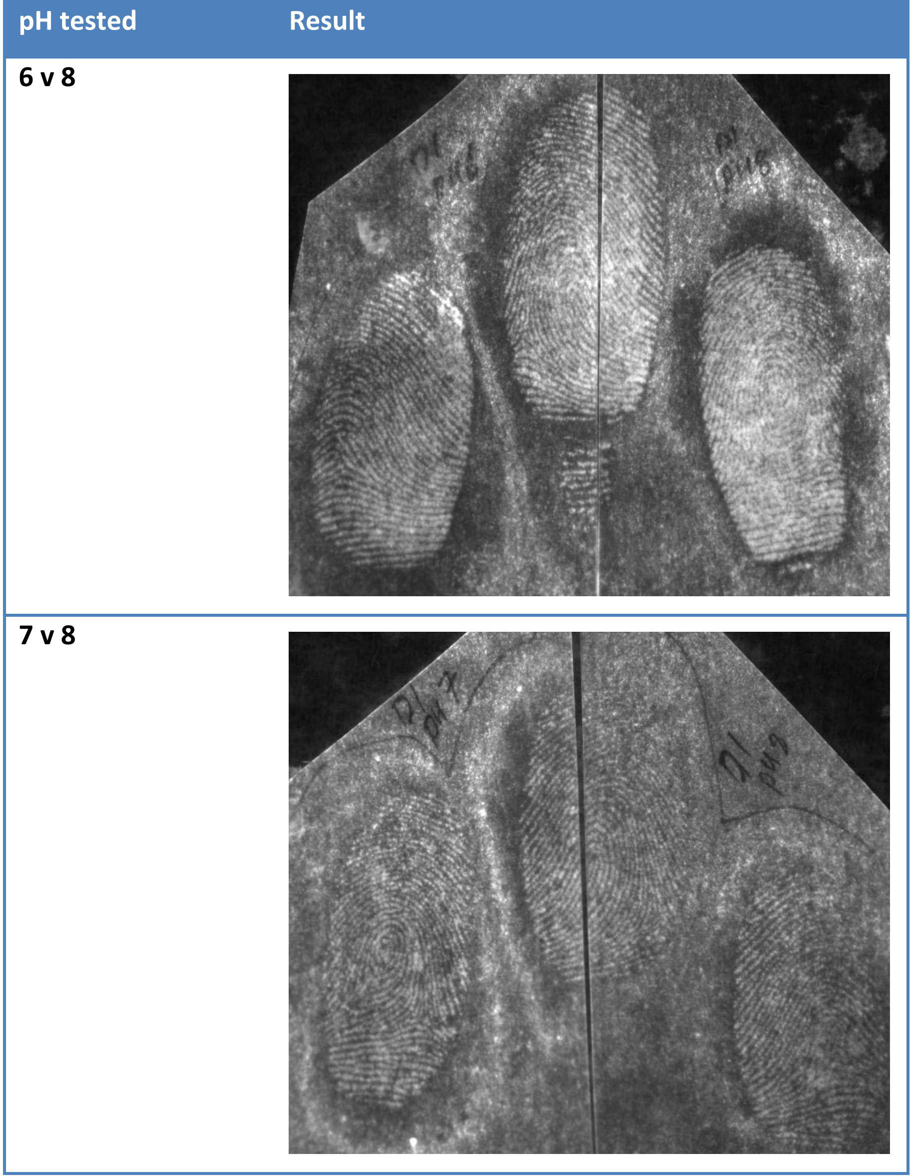


Temperature (^{O}C)	Result
25 v 37	
30 v 37	
40 v 37	

By comparing the results achieved with the different solution temperatures (25 – 40 ^{O}C), it was found at all temperature ranges tested, the development of the latent marks was often similar. At all temperatures, fingermarks were generally always developed with clear ridge detail. It was decided that a temperature of 37 ^{O}C should be used for the remainder of the investigation as this is physiological temperature, and, as such, most bimolecular recognition reactions (including aptamer selection) are performed at this temperature, and, thus would not cause any issues in the degradation of the aptamers.

In the final step in optimising the reagent for use on paper, the pH of the development solution was investigated to see if this would affect development and, if optimised, provide further improvements. The pH levels tested were 6, 7 and 8. pH 8 was used in the reagents optimised in Chapter 4 and was found to work well; this pH helped to keep the aptamer-based reagents stable over long durations. Three development solutions containing aptamer 1 and TBS altered to the correct pH were created, with freshly charged fingermarks subsequently treated. The results are compared in Table 5-3. It can be seen that the different pH levels caused very little difference in the detection and development of the latent fingermarks. With this result verified, a pH of 8 was used for the development solution.

Table 5-3: Results from charged fingermarks freshly placed on paper and developed with different pH levels of the development solution. Fingermarks developed using aptamer 1 (held at 37 ^{O}C) after incubation for 15 seconds. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.



pH tested	Result
6 v 8	
7 v 8	

5.3.2 Collection and storage of fingermarks

As with the previous chapters, the donors used in this section were the same as had been used throughout the research. The use of the same donors therefore meant that there was a good understanding of the general type of fingermarks deposited by each donor.

Fingermark samples containing three-finger impressions were taken from all donors. Donors were asked to place their ring, middle and forefinger down on the paper surface, grouped

together, while the outline of their placement was marked onto the paper with a pencil. Both charged and natural marks were obtained from all donors. Marks were stored in daylight (out of direct sunlight) and protected from contamination by placing samples in a glass tray and covering with a loose fitting cover. Marks were kept at room temperature, which generally ranged from 18–22 ^{O}C, with a relative humidity between 30–55%.

5.4 Materials and methods

5.4.1 General

5.4.1.1 Reagents

DNA aptamers selected to hen egg-white were synthesised by Biosearch Technologies Inc., USA. Fluorescent tag modifications were undertaken during synthesis. Aptamers were received lyophilised and used as supplied.

Hen egg-white lysozyme [CAS 12650-88-3] dialyzed, lyophilized powder ≈100000 units/mg; human lysozyme [CAS 12671-19-1] recombinant, expressed in rice ≥100,000 units/mg; Trizma® base [CAS 77-86-1] ≥99.9% (titration); potassium chloride [CAS 7447-40-7] BioXtra, ≥99%; paraformaldehyde [CAS 30525-89-4] reagent grade, crystalline; zinc chloride [CAS 7646-85-7] 99.999% trace metal basis; glutaraldehyde solution [CAS 111-30-8] grade II, 25% in H_2O; trimethylchlorosilane [CAS 75-77-4] purified by redistillation ≥99%; hexamethyldisilazane [CAS 999-97-3] and 5-sulfosalicylic acid hydrate [CAS 304851-84-1] were obtained from Sigma-Aldrich and used as supplied.

Ethylenediaminetetraacetic acid [CAS 60-00-4] (anhydrous, ≥99%) was obtained from APS chemicals and used as supplied.

Sodium chloride [CAS 7647-14-5] was obtained from Chem-Supply and used as supplied.

1,2-Indanedione [CAS 16214-27-0] was obtained from the Casali Institute and used as supplied.

AR grade glacial acetic acid [CAS 64-19-7] was obtained from Chem-Supply and used as supplied.

Hydrochloric acid [CAS 7647-01-0] (36% w/v) was obtained from Univar Ajax Finechem and used to prepare a 1 M HCl solution.

Ethanol absolute [CAS 64-17-5] and methanol [CAS 67-56-1] were obtained from AnalaR NORMAPUR® VWF and used as supplied.

Acetone [CAS 67-64-1], 1-propanol [CAS 67-63-0], AR grade dichloromethane [CAS 75-09-2], ethyl acetate [CAS 141-78-6], 1-butanol [CAS 71-36-3] and isopropanol [CAS 67-63-0] were obtained from Chem-Supply and used as supplied.

Tetrahydrofuran [CAS 109-99-9] anhydrous, ≥99%, inhibitor-free, was obtained from Sigma-Aldrich and used as supplied.

Novec 3M HFE 7100 was obtained from Novaline and used as supplied.

5.4.1.2 Instrumentation

Reflex 'Ultra White' 80 GSM copier paper was the surface employed for all fingermark samples in this chapter.

A Rofin Poliview IV imaging system with a Rofin PL500 Polilight forensic light source was used for the visualisation of treated lysozyme spots and fingermarks.

A Ratex instruments vortex mixer was used for the mixing of oligo solutions.

A Kelvinator Opal fridge/freezer was used for the storage of DNA aptamers and solutions.

A Julabo ShakeTemp SW22 was used as a temperature controlled water bath.

A Digitech QM1602 digital thermometer was used to monitor the temperature of the development solutions.

A Singer magic steam press 7 was used for the development of fingermarks treated with IND-Zn.

An Eppendorf 5702 centrifuge was used for the spinning down of the oligos.

Aptaca 90 mm sterile polystyrene petri dishes were obtained from Livingstone International.

Gladfoil heavy duty aluminium foil was used to cover the development solution.

5.4.2 Development of aptamer-based reagent for use on paper surfaces

Both aptamer sequences were re-hydrated by using TBS (pH 8) to create an aptamer solution of 0.1 mM. Once TBS was added, the aptamers were vortexed for 1 minute before being centrifuged at 4000 rpm (1431 g) for 30 seconds. To produce aptamer-based development solutions, 30 mL of TBS was added to a sterile petri dish. To the TBS, a 200 µL aliquot of either aptamer was added and stirred. At all times the tubes and petri dishes containing the development solutions were protected from light by using aluminium foil. When solutions were not being used, they were stored in the dark and at 4 ^{O}C.

5.4.3 Aptamer reagent application to paper surfaces

Human and egg-white lysozyme standards were used as detailed in Section 4.4.2. Paper samples were pipetted with 10 µL of the lysozyme standards at concentrations of 1 µg/µL and 0.1 µg/µL. Samples were placed in a clean glass dish and loosely covered. These were left to dry at room temperature for a minimum of 24 hours. Fingermark samples were collected as detailed in Section 5.3.2.

Aptamer-based development solutions were held at a temperature of 37 ^{O}C through the use of a temperature controlled water bath. The petri dishes containing the development solutions were floated on the surface of the water with the use of polystyrene (Figure 5-3). When in use, the water bath containing the development solutions was covered with aluminium foil. Lysozyme and fingermark samples were placed into the developing solutions fingermark side down. Samples were left to incubate for 15 seconds before being removed. Excess reagent was shaken off the samples then the samples visualised using the Poliview with a 505 nm excitation band and a 555 nm band-pass camera filter. Samples that required a post-development wash step were placed in methanol held at 0 ^{O}C for five seconds before being re-imaged.

Figure 5-3: Aptamer-based development solutions held at 37 ^{O}C using a temperature controlled water bath.

5.4.4 Comparison studies to existing techniques

The comparison of development with the aptamer-based reagents with that achieved using IND-Zn was conducted on fingermarks collected as detailed in Section 5.3.2. These marks were cut vertically down the centre of the fingermark impression (as explained in Section 4.3.4). One half of the mark was subjected to the exact aptamer development method as stated in Section 5.4.3. The other half was subjected to IND-Zn treatment following the procedure as stated in the NCFS Fingermark Detection and Enhancement workshop manual [226] (see Appendix II). After development, both halves were visualised using the Poliview with the same settings. Only exposure times were different for each type of development.

5.5 Results and discussion

5.5.1 Performance of aptamer reagent on paper surface

5.5.1.1 Initial performance of aptamer reagent

As has been shown in Section 5.3.1, the aptamer-based reagents required re-optimising before any trials could be undertaken for their use on paper samples. Before fingermarks were investigated, the reagents were re-examined for their potential to detect lysozyme standards that had been pipetted onto paper and left to dry for a minimum of 24 hours. As Figure 5-4 shows, lysozyme standards could be detected on paper, with the reagents still able to detect lysozyme down to 1 ng as had been possible in the previous chapter.

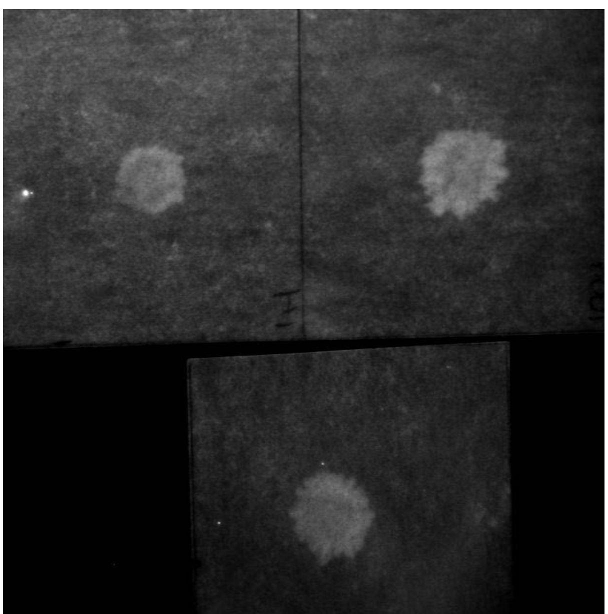


Figure 5-4: Human lysozyme standards on paper developed with aptamer 2 after incubation for 15 seconds (10 µg top left, 1 µg top right, 1 ng bottom). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

Although lysozyme could be detected with clear fluorescent spots resulting, the bright and intense fluorescent visualisation seen with the PVDF membranes was not achievable on the paper samples. The main reason for this can be seen in the above figure in that each lysozyme spot is uneven in its circular pattern. In the work on PVDF, the visualised lysozyme produced well defined and clear circular edges, illustrating that the lysozyme had not

significantly migrated away from the site of placement. In these results on paper, the 'jagged' edge indicates that the lysozyme has migrated through the more porous paper surface. This therefore causes a lower concentration of lysozyme to be present for the aptamers to interact with.

Another possible reason for the weaker detection of lysozyme on paper could be due to the solubility potential of lysozyme in the aptamer-based development reagent. Lysozyme is a highly basic protein with an isoelectric point of 11 [316]. When proteins lack a net charge, the protein molecules generally interact only between themselves and not with water. However, with a strong charge such as with lysozyme, protein molecules are repelled from each other, therefore interacting with water and becoming soluble [317].

Due to the issues encountered and explained in Chapter 4 regarding the development of natural marks on PVDF, it was not surprising that natural marks placed on paper were unable to be detected and developed. All natural marks tested, whether freshly placed or aged, did not produce any ridge detail. Short incubation times (<10 seconds) were used so as to reduce the potential of lysozyme solubilisation; however, this did not improve results, with generally lightly fluorescent paper the overall result (Figure 5-5).

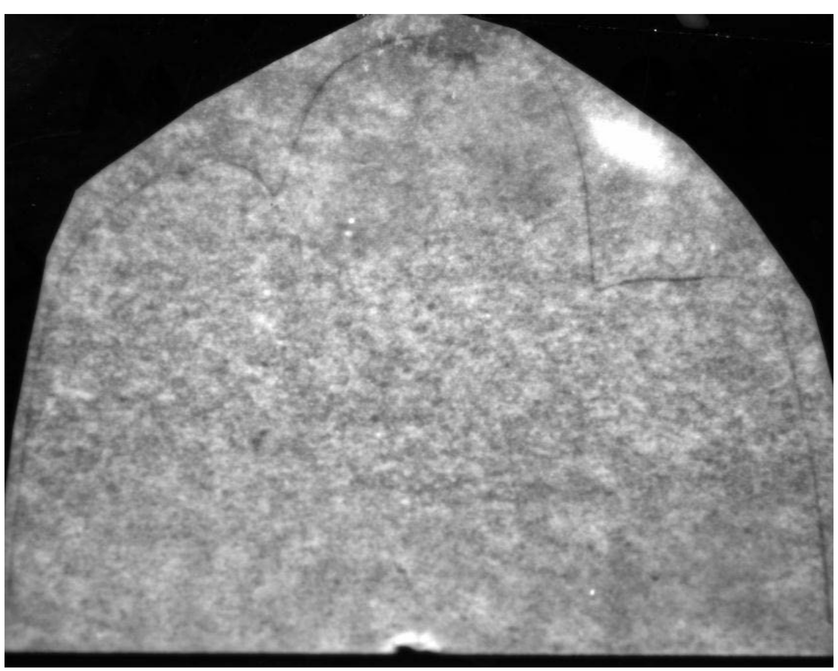


Figure 5-5: Freshly placed natural fingermark after 10 second incubation in aptamer 1 solution. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

While natural marks did not develop any ridge detail or even any indication of mark placement, charged marks were able to be detected and developed with both aptamer-based reagents. However, as Figure 5-6 shows, development was initially not as clear or detailed as had been possible on PVDF membranes. Detail was often weaker, while increases in paper fluorescence reduced contrast between the ridges and the background. As explained with the lysozyme standards, this weaker development is believed to be due to lysozyme migration and solubilisation once in the development solutions. This therefore meant that a suitable incubation time needed to be found that would not cause the lysozyme to be 'washed away' but would still give the aptamers sufficient time to interact with any lysozyme contained within the latent mark.

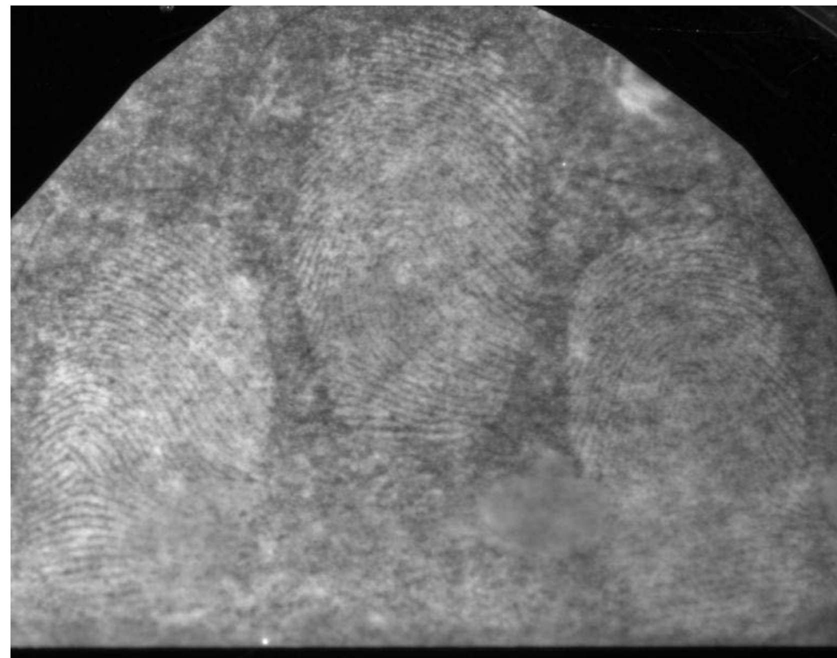


Figure 5-6: Freshly placed charged fingermark developed with aptamer 1 after incubation for 20 seconds. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

Incubation times of 10, 20, 40, 60 and 120 seconds were trialled on a number of charged fingermarks obtained from various donors. For the optimised conditions, it was found that incubation times of 10 and 20 seconds generally produced fingermarks with the most detail visible. Incubation times longer than this often reduced the amount of detail present in the marks, with marks becoming blurred and faint with incubation times of 60 and 120 seconds (Figure 5-7). This blurring and eventual loss of detail from the fingermarks again tended to point to the issue of lysozyme migration and solubility.

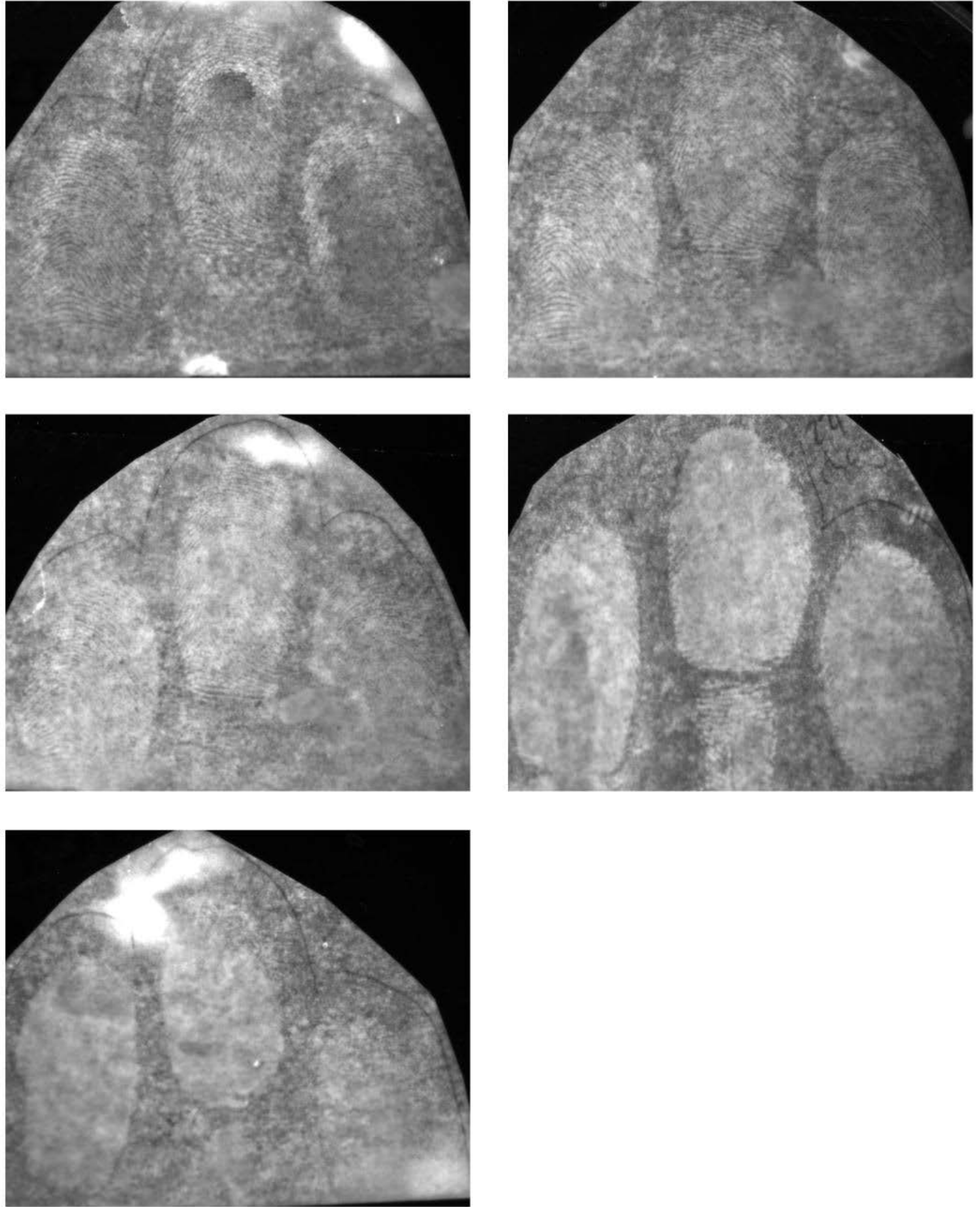


Figure 5-7: Comparison between incubation times on freshly placed charged fingermarks by the same donor and developed with aptamer 2. 10 seconds (top left), 20 seconds (top right), 40 seconds (middle left), 60 seconds (middle right) and 120 seconds (bottom). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

With an optimised incubation time of 15 seconds chosen, both reagents were first tested on freshly placed charged fingermarks from all donors. In these initial trials, however, it was clear that there was no consistency in the results obtained. Development was highly variable on fingermarks obtained from both different donors and from the same donors. All donors used in the study provided fingermarks that did, at least once, develop fingermarks with clear

ridge detail. However, all donors also did, at least once, provide fingermarks that were either blurred, lacked detail or were not visible at all (Figure 5-8).

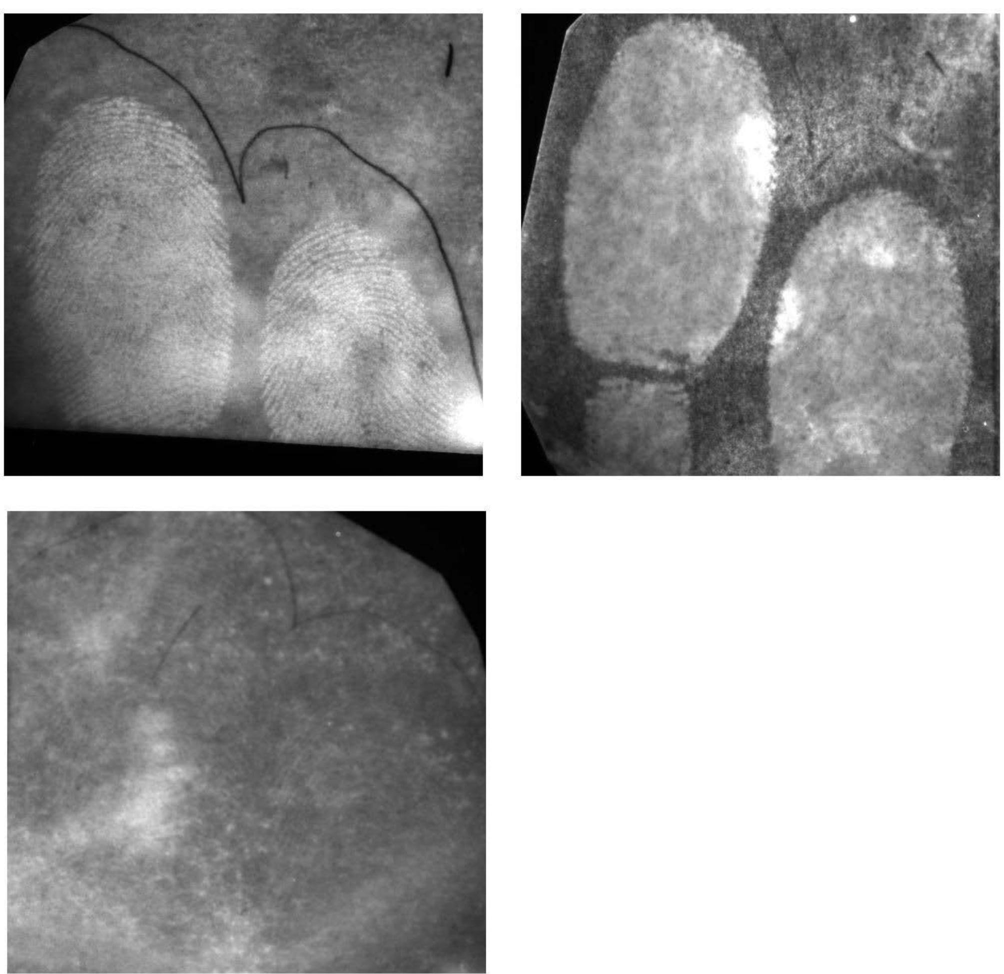


Figure 5-8: Illustration of the variation in development of charged fingermarks from the same donor deposited over the course of 1 week. Marks all developed with aptamer 1 reagent under optimised incubation time of 15 seconds. Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

This variability in results is believed to be due to a number of issues. Marks that did not produce any detail even though they were charged were all obtained from donors in the morning. If those same donors provided marks later in the day, however, fingermarks or at least mark placement patterns could be developed. This highlights the fact that the lysozyme together with the skin's natural oils and secretions need time to build up if they have been removed through contact with water (e.g., as a result of showering). This was supported by certain donors whose fingermarks could be developed clearly even if they were placed in the morning as they had not removed the skin's natural secretions earlier in the morning. The

other main reason for the variability in results is due to the wide differences in fingermark composition between donors. As has already been discussed (see Section 4.5.1), both fingermark composition before placement and its subsequent behaviour once deposited can vary dramatically between donors [32, 233, 243]. While the concentration of lysozyme in healthy human skin is known (see Section 4.3.1), it still remains un-quantified in human sweat and was, until now, an unknown protein in latent fingermarks. This therefore means that, as it is impossible to know the variability in the quantity of lysozyme between donors, results such as these are not to be unexpected.

Marks that were blurred or did not develop full ridge detail were hypothesised to be due to two main reasons. As has already been stated, lysozyme can be a highly mobile and soluble protein when in the presence of water. Even though a short incubation time was thought to be able to provide suitable development without affecting the lysozyme, it may still have been too long. Unfortunately, the use of shorter incubation times on these marks was unable to solve the issue as marks treated in this way provided very weak detail that was hard to visualise with the Poliview system. With certain donors, the other reason for the blurred fingermark development was believed to be due to 'overloaded' marks. As fingermarks were 'charged', these donors often collected far more material on their fingers than was required. When these marks were then placed on the paper surface, this excess material caused ridge detail to be obliterated. This was investigated by undertaking depletion marks from donors who were found to often provide blurred fingermarks (Figure 5-9).

Depletion marks are a series of marks that are placed one after the other without the fingers being re-charged after each placement. As shown in Figure 5-9, the first fingermark to be placed after charging does provide some ridge detail around the side of the mark but detail is quite blurred in the centre of the mark. This is also apparent in the second mark, with strong fluorescence overpowering the mark. However, in the third and fourth placed marks, the ridge detail that could not be visualised in the first two marks is now quite visible, albeit at a reduced intensity due to the overall reduction in fingermark deposit in these two marks.

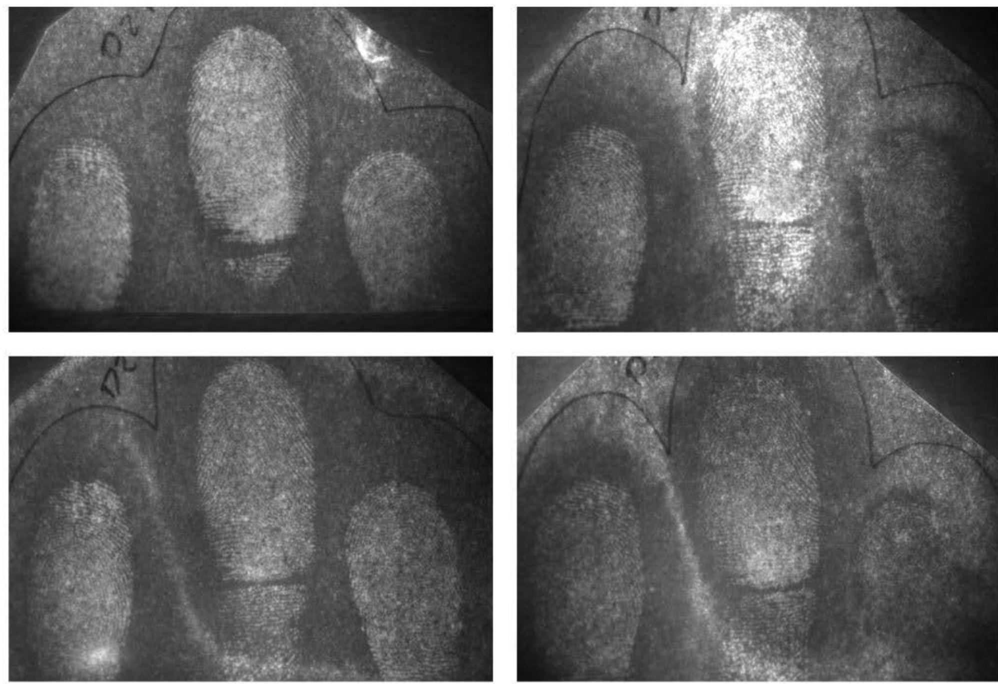


Figure 5-9: Depletion marks from a donor known to provide strongly charged fingermarks. 1st placement (top left), 2nd placement (top right), 3rd placement (bottom left) and 4th placement (bottom right). All marks developed with aptamer 1 with incubation of 15 seconds and visualised in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

5.5.1.2 Optimisation of the development technique for aptamer-based development

With issues surrounding both inter- and intra-donor variability, a method was sought to try and achieve more consistent results. With the quantity of lysozyme available in donor marks appearing to be highly variable, it was felt that a method to try and fix the protein to the paper would allow for longer incubation times, which would give the aptamers longer to interact with fingermarks containing less lysozyme target. A method for fixing lysozyme would also remove the issue of lysozyme solubility and thus give rise to better developed fingermarks.

Protein fixation is a common technique used in biology and, in particular, histology to preserve cells, tissues and proteins prior to undertaking various investigations [318]. Fixatives play many roles, one of which is the ability to render macromolecules (such as proteins) insoluble in water [318]. Chemically, this can be done in one of two ways, either through chemicals that add to the macromolecule allowing it to cross-link (e.g., aldehydes), or

through chemicals that act on the macromolecules without chemically combining with them (e.g., alcohols) [318]. Protein fixation is already used in fingermark detection when enhancing fingermarks in blood. Methanol and a 2% w/v solution of 5-sulfosalicylic acid are the two options generally used to fix blood marks before subjecting them to enhancement procedures [225].

In this work, four different fixing methods were trialled to fix lysozyme in place but still allow for the successful development of latent fingermarks. A 2% w/v solution of 5-sulfosalicylic acid was tested, primarily as it already features in the chemical enhancement of bloody fingermarks, as stated above. Paraformaldehyde and a 4% v/v paraformaldehyde/1% v/v glutaraldehyde solution were tested as both are recommended for use on most proteins and enzymes [319]. Ice-cold methanol was also investigated due to its ease of use and its recommendation by fellow fingermark researchers [320].

Both ice-cold methanol and the 5-sulfosalicylic acid solution were applied to paper containing dried lysozyme standards by placing the samples into the solutions for up to two minutes before being washed with deionised water (5-sulfosalicylic acid only). Once dry, they were subjected to aptamer development in the usual way. Unfortunately, neither of these techniques provided any successful results. The two aldehyde solutions were applied either by placing the items directly into the fixing solutions or by hanging them in an enclosed container containing the solutions. This was to see if direct contact with the fixing solutions would cause any issues. Aldehyde fixing solutions require long fixing times to completely cross-link proteins [318]; therefore, they were left for 24 hours before being washed, dried and developed. Results from the two different solutions were very similar. The samples that were hung over the fixing solutions did produce fluorescent spots; however, the development generated was much weaker than the control samples that had undergone no fixation. One thing to note, however, with the samples that were hung is that they show a reduction in background interaction. This is believed to be due to the paraformaldehyde acting as a blocking agent against the cellulose surface. Although this is beneficial (and investigated later with alternative methods), again due to the reduction in fluorescence this hanging method was not investigated further. The samples that had been in direct contact with the fixing solutions did not produce any fluorescent spots; instead, they produced very dark spots (Figure 5-10).

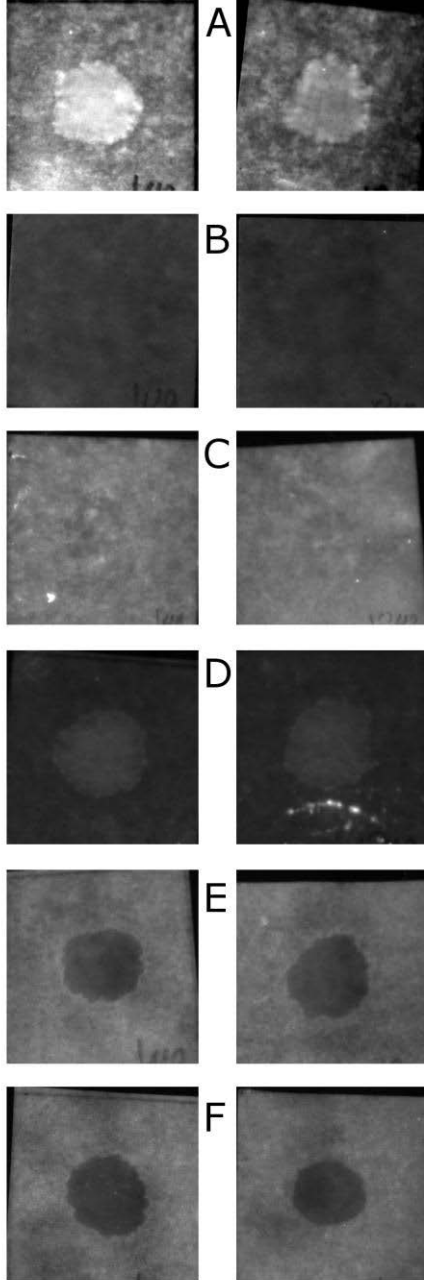


Figure 5-10: Results obtained from different lysozyme fixation strategies on 10 µg lysozyme spots. All developed with aptamer 1 (left column) and aptamer 2 (right column). Control samples (A), ice-cold methanol (B), 5-sulfosalicylic acid (C), hanging method with 4% paraformaldehyde (D), direct contact with 4% paraformaldehyde (E) and direct contact with 4% paraformaldehyde/1% glutaraldehyde (F). Images recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

The reasons why all of the fixing solutions did not work is not completely understood but it is thought that both the methanol and the 5-sulfosalicylic acid techniques actually removed the lysozyme spot from the paper as no evidence of lysozyme's presence could be seen after aptamer development (Figure 5-10, B & C). With the aldehyde solutions, both appear to have

fixed the lysozyme in place due to it being visible on the paper as dark spots (Figure 5-10, E & F). However, the solutions seem to have altered the lysozyme in some way, causing the aptamers to be unable to interact successfully. Formaldehyde has been shown to cause no effect to the secondary structure of certain proteins, with research stating that it 'locks in' the secondary structure of the proteins tested [321]. However, aldehydes are known to change both the chemical and antigenic profiles of proteins due to the introduction of some artefact in order to provide a protective effect [318]. This change could therefore be the reason for why the lysozyme can actually be seen on the paper surface but is not detected by the aptamer.

With the fixation of lysozyme failing to improve on results obtained with the aptamer-based reagents, other areas that could provide improvements in development were investigated. With both aptamer reagents, it was found that although fluorescent ridge detail was achievable it often resulted in a fluorescent background being developed; hence, contrast between the fingermark and the paper background was generally reduced. The reason for the background fluorescence was believed to be due to hydrogen bond interactions between the hydroxyl groups of the cellulose in the paper and the phosphate backbone of the DNA. In order to investigate this further, two silylation agents were used to derivatise the hydroxyl groups contained within the cellulose.

First, a 5% v/v solution of trimethylchlorosilane (TMCS) in dichloromethane (DCM) was tested on blank pieces of paper. Paper was placed into the solution for between 10 to 120 minutes before being dried and tested in either aptamer reagent. With just a five minute submersion in TMCS, results showed a significant reduction in background fluorescence. Silylated paper produced backgrounds with no fluorescence, whereas the control samples that had not been treated with TMCS were strongly fluorescent (Figure 5-11).

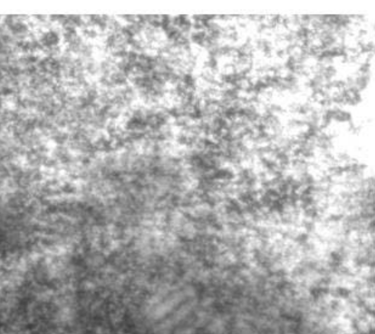


Figure 5-11: Paper samples after incubation for 15 seconds in aptamer 2. Silylated paper (left), untreated paper (right). Images recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

With these results, TMCS was then applied to paper surfaces containing charged fingermarks and lysozyme standards. After development with the aptamer-based reagents, it was found that, with the lysozyme standards, TMCS was successful in reducing the background fluorescence considerably compared to control samples, which led to much clearer development of the lysozyme spots (Figure 5-12). However, the TMCS was not as successful on charged fingermarks. Although background fluorescence was reduced, no fingermark detail could be developed (Figure 5-13). The reason for the lack of fingermark detail after TMCS treatment is likely due to the use of DCM as the solvent for TMCS. DCM is a slightly polar solvent, therefore the charged fingermark, which contains various fingermark constituents in an emulsion of hydrophobic fatty acids and lipids, is stripped from the paper surface.

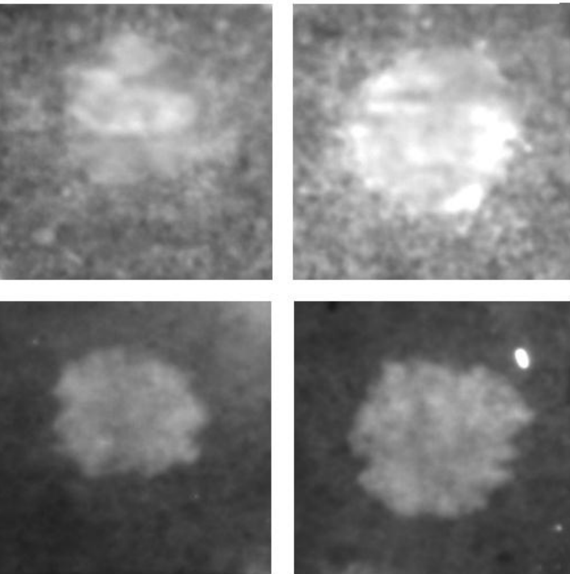


Figure 5-12: Lysozyme standards (1 µg) after development with aptamer 1 (left) and aptamer 2 (right) after incubation for 15 seconds. Untreated paper (top) silylated paper (bottom). Images recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

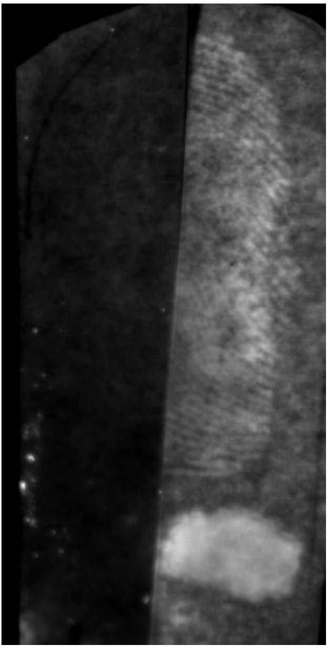


Figure 5-13: Charged fingermark developed with aptamer 1 after incubation for 15 seconds. Silylated paper (left), untreated paper (right). Images recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

In order to try and combat this issue, a second silylation agent and carrier solvent was investigated. Hexamethyldisilazane (HMDS) diluted to 2% v/v in tetrahydrofuran (THF) was used in the same way on charged fingermarks. THF was used to try and overcome the issue of

removing the fingermark deposit, as THF is one of the most polar ethers available. Once developed, however, it was found that HMDS was not as successful as TMCS in blocking the background, with all results showing strong background fluorescence with no fingermark detail visible (Figure 5-14).

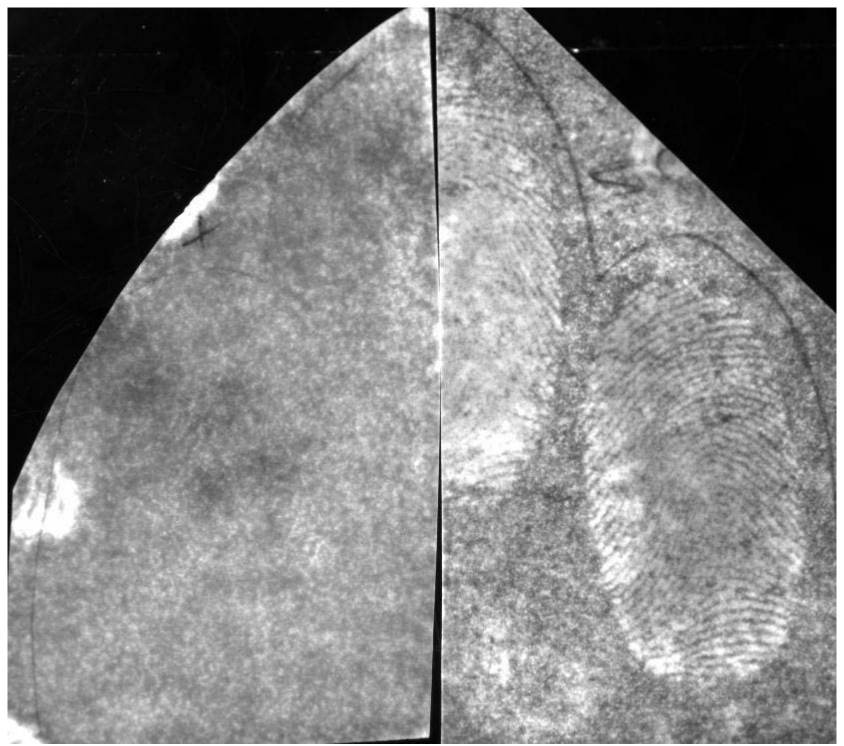


Figure 5-14: Charged fingermark developed with aptamer 2 after incubation for 15 seconds. Silylated paper with HMDS (left), untreated paper (right). Images recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

Although the use of either of these reagents was found to be unsuitable in this current work, it did prove that there is a non-specific interaction between the cellulose substrate and the DNA backbone. Unfortunately, this interaction is one of the main causes for the reduction in contrast and ridge detail seen on the paper samples developed by the aptamer-based reagents. Without this interaction, fingermark detail is far more clear and detailed as shown in Figure 5-15. In this figure, charged fingermarks were placed onto paper that had been pre-treated with TMCS and left to dry. As Figure 5-15 shows, the section of the paper that has been treated produced fingermarks that were clear, with level 2 details easily visible, whereas the side without pre-treatment is strongly fluorescent, which overpowers the developed ridge detail.

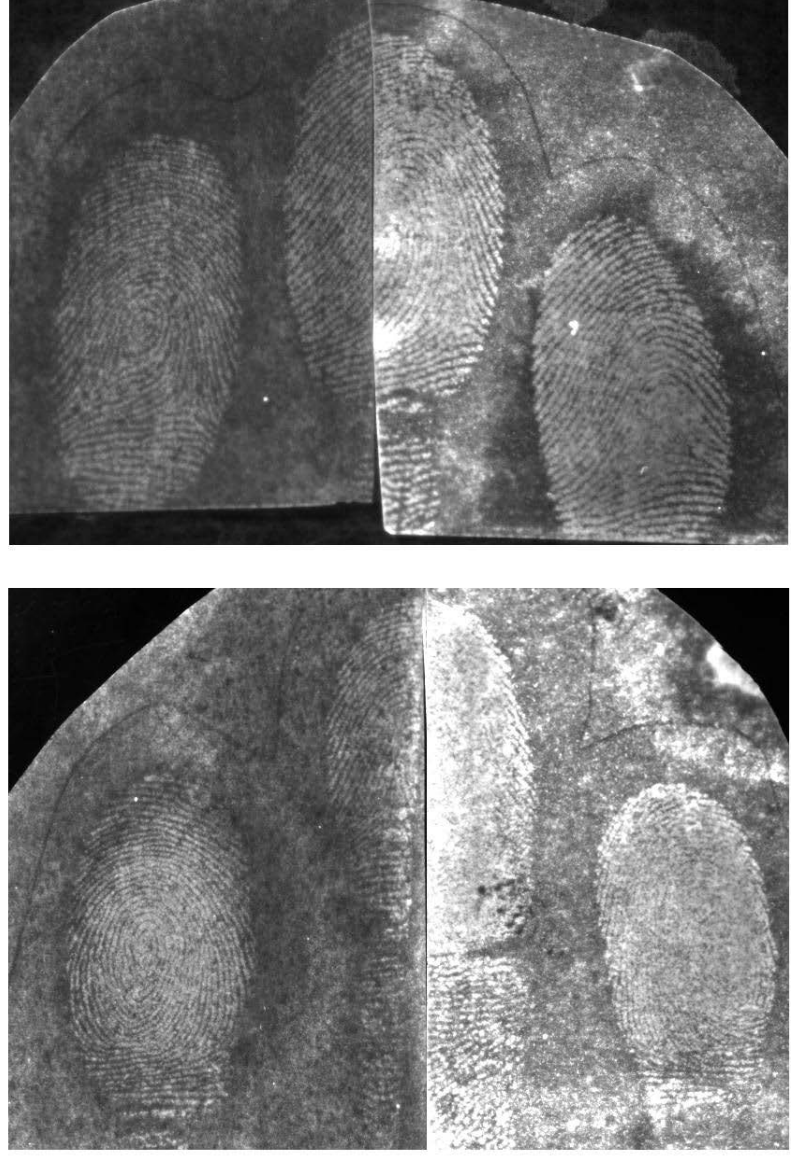


Figure 5-15: Charged fingermarks (different donors) after development with aptamer 1 using an incubation of 15 seconds. Pre-treatment of paper with TMCS before mark placement (left), untreated paper (right). Images recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

As previously stated, a main issue with the aptamer-based reagents used on paper surfaces is the non-specific interaction with the cellulose substrate causing fluorescent backgrounds that, sometimes, are so strong that it overpowers any ridge development. As blocking methods were found to be incompatible with this work, post-development washing steps were investigated to see if some of the background staining could be removed and therefore improve the contrast of the developed fingermarks.

A methanol post-development wash step was first investigated due to its success as a post development wash for the RNA-based reagents (see Chapter 3). After normal aptamer development, samples were placed directly into the methanol for five seconds before being visualised with the Poliview. Results did show a reduction in background staining after the methanol wash but fingermark detail was also reduced. To overcome this, the methanol was cooled to 0 ^{O}C therefore reducing the polarity of the solvent. Aptamer developed fingermarks washed in ice-cold methanol for five seconds were found to result in a significant reduction in background staining but still produce clear and detailed ridge detail as seen in Figure 5-16.

The one drawback with using methanol as a post-development wash step, however, was that it can cause certain inks to run when in direct contact. Obviously, this would be a problem when processing paper evidence items marked with ink (e.g., bank cheques). In order to find an alternative solution, a number of alcohols and ketones were tested for their ability to remove background staining, maintain fingermark detail, and stop ink running. Those tested included butanol, propanol, iso-propanol and acetone. All of the solvents were tested both at room temperature (RT) (approx. 20 ^{O}C) and at 0 ^{O}C. Results from these trials can be seen in Table 5-4.

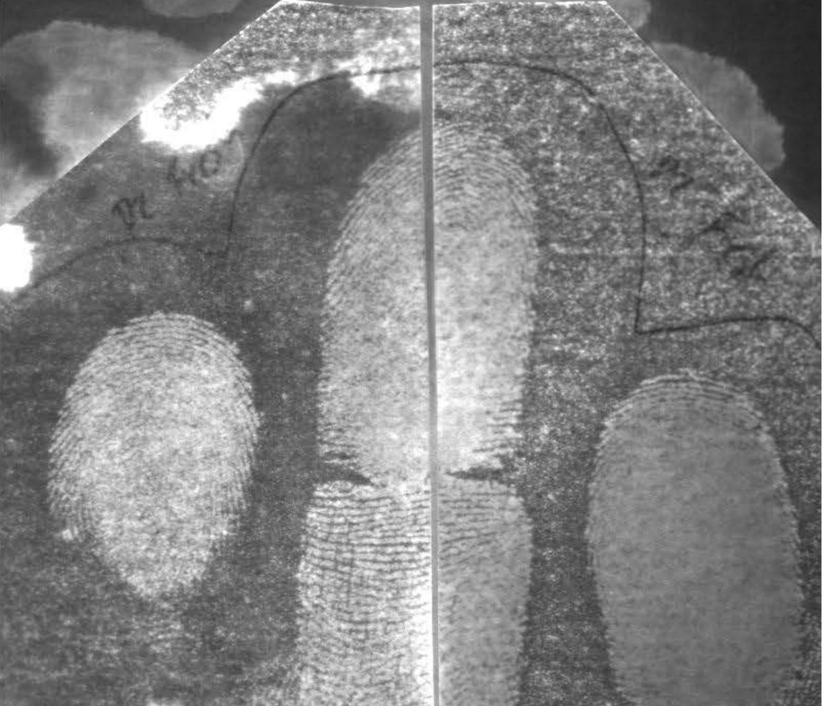


Figure 5-16: Charged fingermarks (different donors) developed with aptamer 1 after incubation for 15 seconds. After post-development wash in ice-cold methanol (left) and no wash (right). Images recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

Table 5-4: Results from the use of different solvents on the removal of background fluorescence and ability to maintain ink detail.

Solvent	Fingermark detail intact	Background fluorescence removed	Ink unaffected
Butanol RT	Yes	No	No
Butanol 0 ^{O}C	Yes	No	No
Propanol RT	Yes	No	No
Propanol 0 ^{O}C	Yes	No	No
Isopropanol RT	No	Yes	No
Isopropanol 0 ^{O}C	Yes	Yes	No
Acetone RT	No	Yes	No
Acetone 0 ^{O}C	No	Yes	No

As Table 5-4 shows, none of the other solvents tested were able to provide results where the ink was unaffected. This is a problem if dealing with marks on documents that contain ink entries. For this study, however, the aim was to provide an initial investigation into the potential of lysozyme aptamers for the detection of latent fingermarks on paper. This has been achieved. Further investigation would need to look at removing the need for a wash step by undertaking various SELEX and aptamer modifications to remove the issue of the non-specific binding with cellulose.

As was seen in Chapter 4, results on marks that were over three to four days old were very poor, with many treated marks either producing very little detail, mark placement patterns only, or with no detail at all. Paper samples were again tested as in Chapter 4 with marks being placed onto the paper and then being stored at room temperature, 4 ^{O}C and -20 ^{O}C. Results from these trials, not surprisingly, followed the trend as seen in Chapter 4. Again, marks were stored for up to 30 days under the different storage conditions before being developed with either aptamer reagent. It was found that, although detail was often reduced when compared to the results achieved on PVDF (see Section 4.5.1), marks always produced better detail when stored under cooler conditions (Figure 5-17 and Figure 5-18). Again, this is

believed to be due to the degradation of lysozyme by other components present in the fingermark, while the overall reduction in detail is also due to the migration of lysozyme into the paper layers.

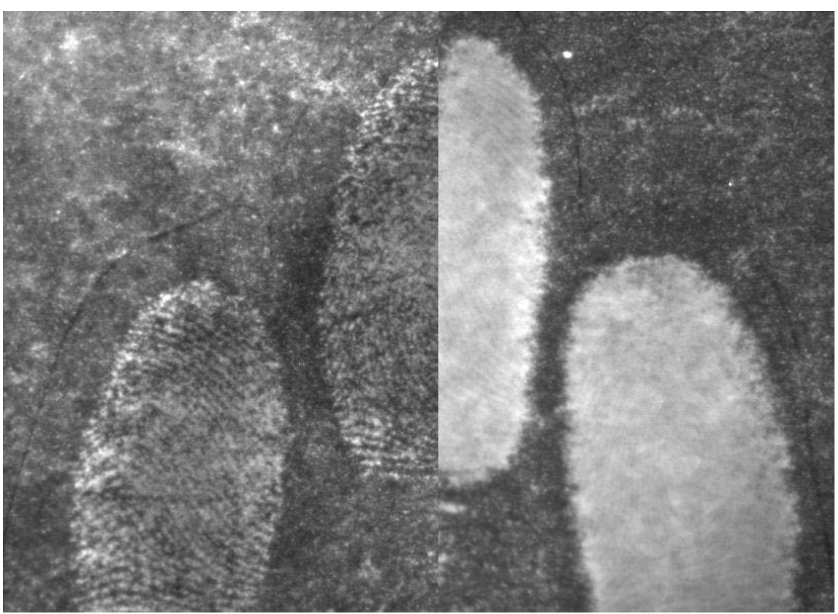


Figure 5-17: Charged fingermarks aged for 30 days and developed with aptamer 1 after incubation for 15 seconds. Two different storage conditions used, 4 ^{O}C (left) and room temperature (right). Images recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

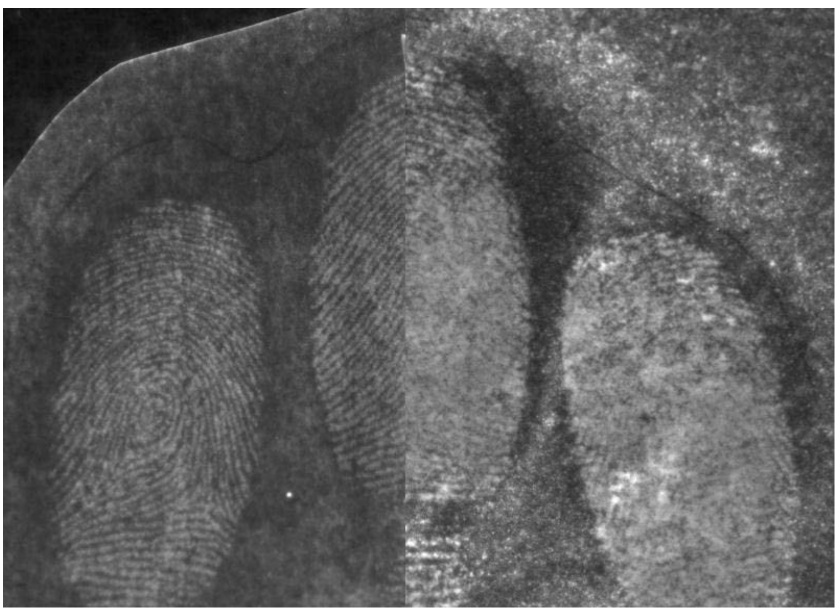


Figure 5-18: Charged fingermarks aged for 30 days and developed with aptamer 1 after incubation for 15 seconds. Two different storage conditions used, -20 ^{O}C (left) and room temperature (right). Images recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

5.5.2 Relative performance of the aptamer reagent against a commonly employed porous surface fingermark technique

While the aptamer-based reagents have produced some relatively good results, it is necessary to relate the current performance of the aptamer-based reagents with reagents currently used for routine latent fingermark detection. Although a full study looking into age and other fingermark variables is recommended when comparing new techniques to current techniques, it was felt that this would not provide useful data in this study. This is because, as has already been shown, the aptamer-based reagents suffer with fingermarks that are anything but charged and relatively fresh (aged for no more than two to three days). Obviously, this does not make the technique a suitable and useful method for the vast majority of fingermark enhancement cases. However, for the purposes of comparison and to explore its current level of performance, a comparison against IND-Zn was nevertheless undertaken.

IND-Zn was chosen as it is one of the most commonly used fingermark development techniques for porous items, with many studies highlighting it as an excellent fingermark

reagent [225]. IND-Zn reacts with amino acids contained within the fingermark as detailed in Section 1.1.7.2. Obviously this interaction with the fingermark is different to the aptamer-based reagents that do not target amino acids but rather proteins. However, IND-Zn will allow for a comparison with a very successful routine technique and provide good data into the relative performance of the aptamer reagents.

As previously stated, only freshly placed charged fingermarks were investigated from four donors. Donor fingermarks were split vertically down the centre to allow for direct comparison of the two reagents on the same placed mark. Both reagents were utilised under optimum conditions before results were visualised on the Poliview system where only exposure times were altered for each technique. For the purpose of this investigation, only aptamer 1 was used due to the fact that both have produced very similar results throughout the course of this research.

As can be clearly seen (Figure 5–19), results from these trials show development by IND-Zn to be far superior to that achieved by the aptamer-based reagent. Detail with IND-Zn is far clearer, resulting in fingermarks where full ridge detail with level 1 and 2 features can be seen. With the aptamer-based reagent, however, detail is less clear, with areas missing detail and a strong background (in comparison with IND-Zn) causing poor contrast. These results are no surprise due to the facts stated earlier with regards to different fingermark components being targeted. Amino acids have been a regular target for fingermark detection for over 50 years, with many reagents being developed that are able to produce the clear detail as seen in the above results. Although lysozyme is contained within a fingermark, this is the first time it has been targeted and, unfortunately, it is still an unknown in terms of the quantity available and how well it interacts with the aptamer-based reagents. As has already been stated, the relative performance of the aptamer-based reagents could most likely be improved through the targeting of different fingermark components that are more abundant and much more stable than lysozyme appears. Through the use of different aptamers, the performance of the reagents is likely to be improved greatly.

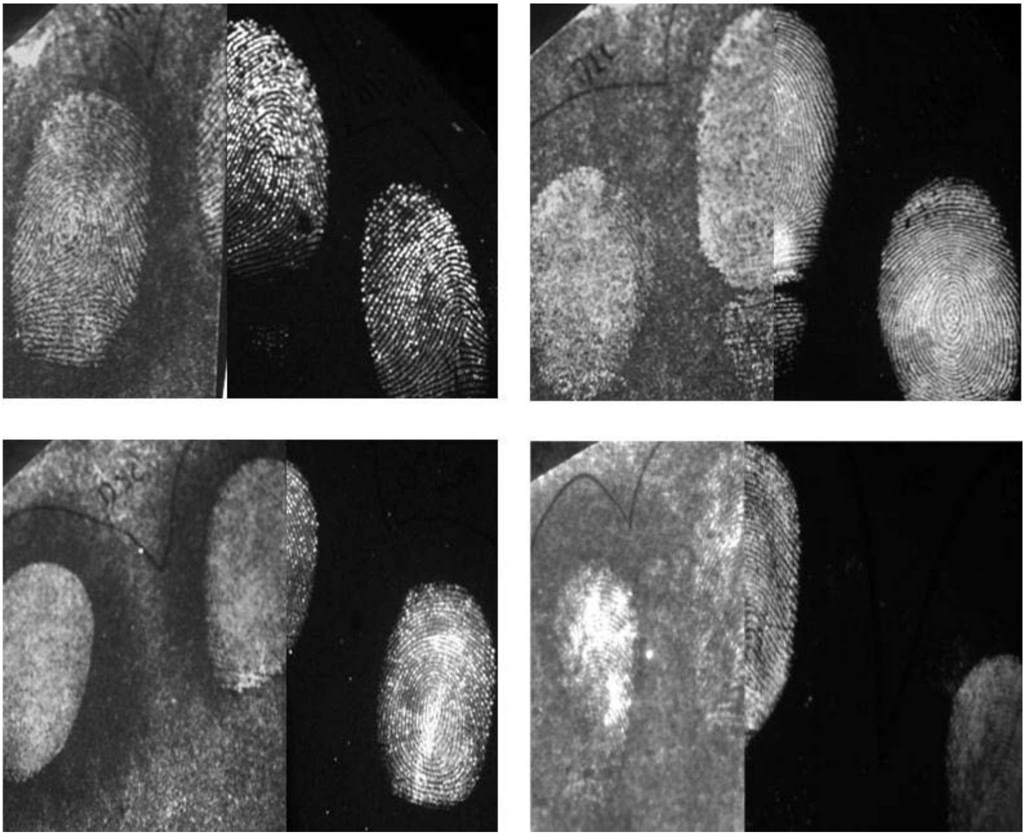


Figure 5-19: Results obtained from various donor comparisons between aptamer 1 development (left) and IND-Zn (right). Images recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

5.6 Conclusion

The aim of this chapter was to advance on work achieved in Chapter 4 with the lysozyme aptamer-based fingermark reagents and investigate their potential for the development of fingermarks placed on plain white copier paper. Although the previous chapter demonstrated the ability of aptamers to detect and enhance latent fingermarks, it was only on a specialised surface not normally encountered in fingermark enhancement laboratories.

The chosen surface (plain white copier paper), is a surface that is far more commonly encountered in fingermark enhancement cases. The overall method used to detect latent fingermarks on paper samples was based on the technique developed in Chapter 4. However, it required re-optimisation due to the greater porosity of paper compared with PVDF, which caused the lysozyme protein to absorb into the paper layers thus making it harder to detect. It was also found that, instead of lysozyme being stable on PVDF due to its protein retention

properties, the protein was very mobile on paper and would dissolve out of the paper if developed for the long incubation periods used with PVDF.

With this, the reagent was optimised for use on paper by increasing the aptamer concentration of the development solution and holding the aptamer developing solution at a temperature of 37 ^{O}C. These optimisations allowed for much shorter incubation times of 10 to 20 seconds to be used rather than exceeding one hour. With shorter incubation times, it was found that the lysozyme would remain intact on the paper surface and this allowed for successful development of both the lysozyme standards and latent fingermarks.

Although latent fingermarks were able to be developed with clear level 2 detail, results were very donor dependent, with significant inter- and intra-donor variability being observed. One reason for the varying results was found to be due to the lack of secretions available from donors who had removed the skin's natural secretions via activities such as showering, for example. However, this was only one potential reason for the highly variable results. It would appear that lysozyme is not always present on the surface of the skin in the same quantity and varies significantly between donors. In order to allow for longer incubation times in the aim of developing better detail, an attempt to fix lysozyme to the paper surface was trialled. Unfortunately, all four different strategies failed either to fix lysozyme in place or allow the aptamers to successfully interact with the lysozyme.

A further issue found with the detection of lysozyme in fingermarks on paper was background interactions between the aptamer and the cellulose surface. Although incubation times were very short, it was often found that the paper surface would become slightly fluorescent after development and hence lead to poor contrast between the developed ridge detail and the paper background. The reason for this was determined to be due to an interaction between the phosphate backbone of the DNA-based aptamers and the hydroxyl group of the cellulose. Attempts to block the hydroxyl group of the cellulose were trialled with two silylation agents. Although these did work in stopping the interaction between the cellulose and the phosphate backbone and thus stop background fluorescence, the necessary solvents for the silylation agents were found to strip the fingermark off the paper, resulting in no fingermark detail being visualised.

The use of an ice-cold methanol wash, however, was found to successfully remove the background staining while leaving the developed fingermark detail intact. This therefore

improved the contrast between the mark and the background, resulting in greater fingermark detail from all donors. With this improvement in development, the aptamer-based reagent was tested against IND-Zn to evaluate the current level of detail developed with the aptamer-based reagent compared to development with an existing fingermark reagent. These trials clearly showed the weakness of the aptamer reagent, with IND-Zn developed fingermarks being far clearer and with much greater detail than could be achieved with the aptamer developed fingermarks.

In conclusion, the work achieved in this chapter has demonstrated the ability of the aptamer-based reagents to develop latent fingermarks on a realistic, casework surface via the targeting of lysozyme. This work has successfully explored and accomplished the overall aims of the project by demonstrating the current ability of aptamers for latent fingermark detection and visualisation through the comparison of the reagent with IND-Zn. Unfortunately, results have been found to be highly variable, with only freshly placed charged marks being able to be fully developed. This is obviously a major issue as the majority of fingermarks to be detected in casework will not be freshly placed and are unlikely to be heavily charged with sebaceous material. However, with the ability of aptamers to be selected to many other fingermark components, it is hypothesised that the use of aptamer reagents to components in greater abundance and with better stability will allow for fingermarks to be developed with greater detail than has been achieved here. Further research is required to fully explore the potential of aptamers as reagents for the detection of latent fingermarks.

Chapter 6: General conclusions, recommendations and future work

Chapter 6: Overall conclusions, recommendations and future work

Although fingerprints have been used for the identification of individuals for over 110 years, they still remain as one of the cornerstones for individualisation in forensic science. In terms of utilising fingermarks from crime scenes and items of evidence, fingermarks are mostly latent and, hence, have to be subjected to one or a sequence of development techniques in order to achieve a contrast between the fingermark ridges and the background on which the mark is placed. Although there are a plethora of latent fingermark development techniques currently available, the field still suffers with a number of issues, including insufficient sensitivity. Leading researchers have highlighted the fact that, with the use of current techniques, a non-negligible number of marks remain undetected (see Chapter 1).

Aptamers have been developed over the past 20 years as an alternative to antibodies for the highly selective and sensitive detection of various analytes (see Chapter 2). Aptamers are developed from either ssDNA or RNA, and are identified through iterative selection cycles in a process called SELEX. Through this approach, aptamers can be engineered and modified to enable them to bind to almost any chosen target. Aptamers have been shown to possess, and in some cases surpass, binding affinities displayed by antibodies to the same target, while they are also easily modified to highlight binding to a target through a number of methods including the use of fluorescent tags.

Although fingermark research has focussed on developing new techniques in order to overcome current issues, including, the lack of sensitivity, progress remains slow with only incremental results generally being achieved. In order to provide more transformational results with respect to sensitivity and selectivity, this project explored the use of aptamers. Although aptamers have displayed great benefits and potential in a wide variety of detection based systems (see Section 2.5), they have, surprisingly, never been investigated as the basis for a latent fingermark reagent.

As amino acids are staple components in the composition of latent fingermarks, the first investigation of aptamers for the detection of latent fingermarks was based on the targeting of amino acids (see Chapter 3). Aptamers selected to the amino acids L-histidine and L-

isoleucine were identified from the literature as suitable candidates for the targeting of amino acids within latent fingermarks. In order to provide a mechanism for visualisation, both aptamers were modified with the attachment of a fluorescent tag compatible with current forensic imaging systems. In this initial work, the full potential of aptamers for the detection of both amino acids and latent fingermarks could not be exploited due to a non-specific dye-secretion interaction (see Section 3.5.4). Although this was obviously an unwanted effect, it did provide a number of answers and a clear direction for the continuation of the project. This included an insight into the use of fluorescent tags, the aptamer binding affinities required, and methods for reagent application.

For the benefit of the project's overall aims, the issues encountered in Chapter 3 meant that the use of amino acids as a target for fingermark detection via aptamer binding was not further investigated. The use of amino acids as a target for aptamers, however, could be re-investigated if access to a complete SELEX selection process were possible. It is believed that, with the ability to undertake a SELEX selection to various amino acids, aptamers could be selected that have far stronger binding affinities than the aptamers available for this project.

Another major issue in this part of the project was the fact that the aptamer reagents needed to be solubilised in methanol with the aid of PEG (methanol was needed as amino acids would be solubilised if aptamers were applied in an aqueous solution). This use of PEG may have interfered with the binding capabilities of the aptamers. This issue could be resolved by undertaking SELEX selections in methanol or other suitable organic solvents. This would allow for the use of aptamers directly in an organic solvent, without the need for PEG or any other method for dissolution. It is, therefore, recommended that this is one area of future work that should be investigated given that amino acids are such a versatile target in latent fingermarks. With the use of more suitable aptamers, results would be expected to be significantly enhanced, without the issues encountered in this project.

By changing the target molecule in latent fingermarks from amino acids to the protein lysozyme, far better results were achieved, with the project demonstrating, for the first time, the detection of latent fingermarks through the use of an aptamer-based reagent (see Chapter 4). A change in target allowed for aptamers to be used that had far greater binding affinities (1000 fold improvement) than had been possible when targeting amino acids. It was found that, from lysozyme standards, lysozyme could be easy detected down to 1 ng on both PVDF and on plain white copier paper (see Chapters 4 and 5). Although fingermarks were

better detected on PVDF, they could still be detected on paper; however, results were less favourable due to diffusion and the lack of retention of lysozyme by the paper substrate (see Section 5.3.1).

The main issue encountered with the targeting of lysozyme for the development of latent fingermarks was that lysozyme was found to degrade relatively quickly, resulting in the aptamer-based reagents being unable to detect latent fingermarks that were older than a couple of days. It was also found that lysozyme was only present in charged marks and not pure eccrine marks (see Section 4.5.1). For the purpose of this project, lysozyme provided the answer that aptamers can be used for the detection of latent fingermarks; however, results would need to be vastly improved before such an approach would be feasible for routine casework.

In future work, the use of other fingermark components as targets for aptamers would potentially be far more suitable. As shown by Reinholz [128], the serum protein albumin is a stable protein for detection, with fingermark samples being able to be clearly detected after 130 days through the use of an antibody-based reagent. Albumin is a major protein found in eccrine sweat and therefore it is assumed to be present in latent fingermarks in higher quantities than lysozyme. Unfortunately, no aptamers currently exist to albumin. Thus, in future work, the selection of aptamers to this protein would need to be undertaken. It is anticipated that, if this protein was to be targeted with an aptamer-based reagent, fingermarks would be developed even if they were aged and uncharged (i.e., low in sebaceous content).

A further issue encountered with the aptamer-based reagents when used on the paper surface was that the phosphate backbone of the DNA-based aptamers interacted through hydrogen bonding with the cellulose surface of the paper. This caused the aptamer-based reagent to become attached to the background and, therefore, reduce the contrast obtained from the development (see Section 5.5.1.2). Although this was overcome to some extent with the use of an ice-cold methanol wash step, further work should investigate the potential to engineer the DNA aptamers and cap the phosphate backbone to prevent this interaction.

Aptamer-based reagents could be developed that do not target just one fingermark component, but several (selected from available eccrine and sebaceous material in the deposit). In the forensic field, this could lead to a far greater potential for developing all

available latent fingermarks with one treatment rather than an extensive sequence of methods. A multi-aptamer reagent could limit the need for the sequencing of reagents and be used as a 'multi-purpose' reagent, therefore reducing overall development times and the potential to damage marks due to the number of reagents used.

The benefit of the use of aptamers is that virtually any target molecule can be selected to provided that the correct selection strategies are applied. Although the main aim of this project was to investigate the potential of aptamers for the detection and visualisation of latent fingermarks, aptamers could be used to provide 'extra' information from those latent fingermarks. As has already been demonstrated in work by Russell and co-workers [124-127], information regarding the use of drugs can be obtained from latent fingermarks through the use of an antibody-based reagent, while at the same time, develop clear fingermark detail. This is an area where aptamers could very easily be used and it is recommended that this be an area considered for future work. This work could focus on two areas, as described below that currently are major problems for law enforcement agencies around the world.

Firstly, as terrorist actions throughout the globe continue, forensic scientists are continuously searching for greater forensic evidence at scenes of crime and from individuals suspected of carrying out terrorist attacks. One area where the use of aptamers for fingermark detection could be used is in identifying if fingermarks from a suspect present any information regarding recent contact with explosives. Currently, an aptamer to trinitrotoluene (TNT) has been developed that could be used in preliminary trials without the need to undertake any specific SELEX selections [241]. Aptamers to a variety of explosives could then be developed for the specific detection of individual explosives.

A second area currently receiving global attention is the use of doping agents by professional athletes to gain a competitive advantage over their rivals. Drug detection in sport has been undertaken for many years; however, with the rapidly changing drug market, the novel methods used by athletes to ingest these drugs, and the time to undertake drug testing, governing bodies can struggle to quickly identify athletes using performance enhancing drugs. One possible simple and quick method to test athletes at competitions could through the collection of a fingermark from the athlete and the subsequent development of the fingermark with a reagent containing aptamers targeted to specific drug metabolites. By targeting specific metabolites, any developed fingermark would therefore highlight the

consumption of a drug by the athlete. In addition, the fingermark itself can be used to conclusively identify the individual who provided the sample.

A 'multi-purpose' reagent could also be used when using fingermarks for gaining intelligence. By producing a reagent with numerous aptamers targeted to different molecules, quick and efficient preliminary analysis of the fingermarks could be undertaken. Each aptamer targeted to the different molecules could be modified with fluorescent tags of various different wavelengths. Thus, when imaged under different lighting conditions visualisation of the fingermarks at specific wavelengths would highlight the presence of the exact molecule.

The main objective of this project was to provide a proof-of-concept study for the use of aptamers as a method for the detection of latent fingermarks. The aptamer-based reagents were found to easily detect lysozyme at 1 ng, highlighting the high sensitivity of the reagents. The selectivity of the reagents was also demonstrated through their lack of affinity to several fingermark components and commonly encountered proteins. It was found, however, that lysozyme was not the most suitable target for use in this project due to degradation issues. However, due to limited aptamer availability, and being unable to undertake SELEX selections, lysozyme was the only target that could be used for this project. Overall, the project did demonstrate the possibility of aptamers to detect fingermarks through the detection of lysozyme, producing clear ridge detail in most of the freshly placed marks that were treated. With this work, it can be seen that by selecting aptamers to other, more suitable fingermark components, and through the modification of the aptamers used, latent fingermark detection with increased sensitivity and selectivity could potentially be achieved.

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Appendices

Appendix I - Additional donor results from the effect of storage conditions on fingermarks placed on PVDF.

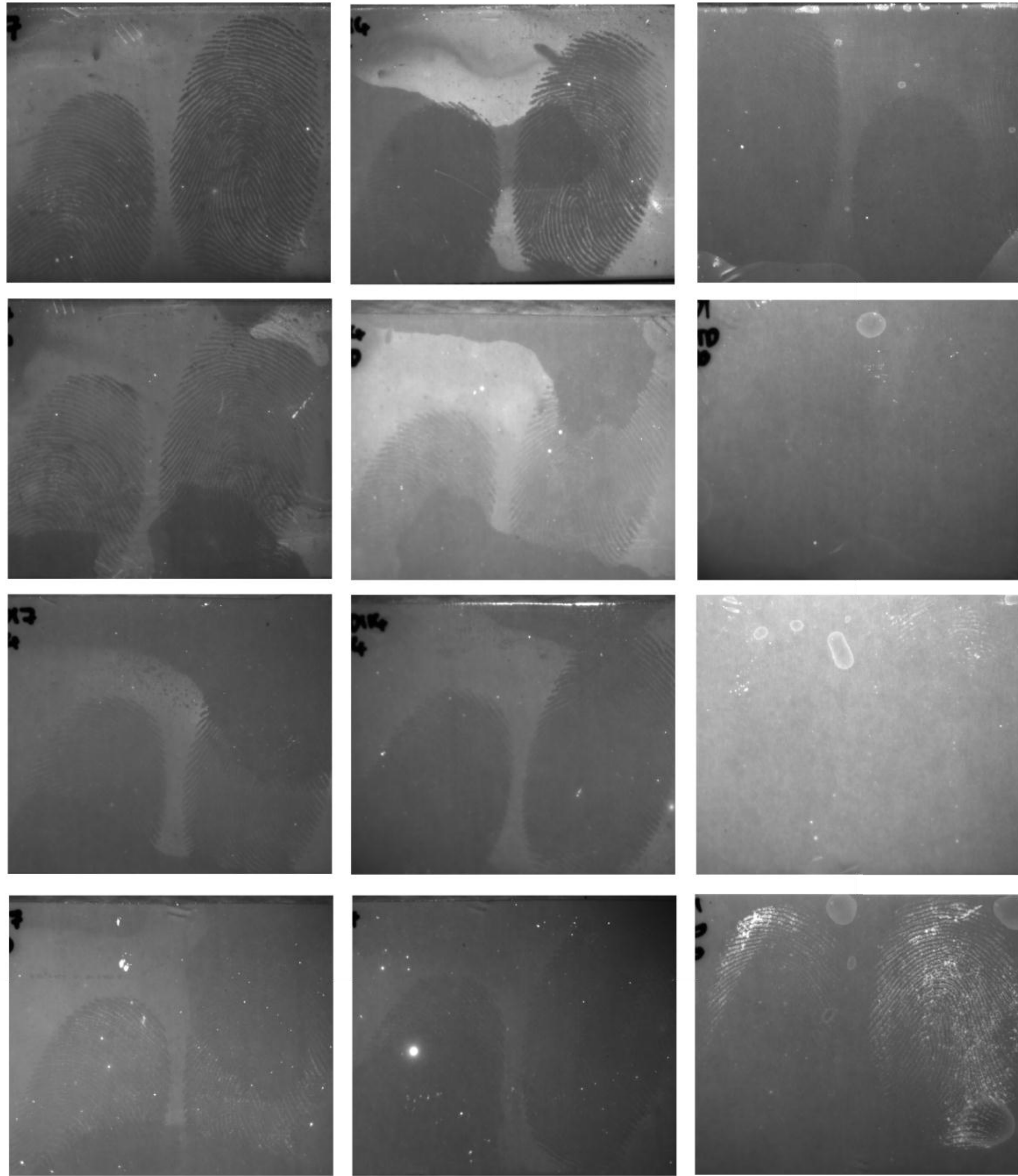

Figure A-1: Results from the effect of storage conditions with charged fingermarks from donor 1 developed with aptamer 1. Seven day aging (left column), 14 day aging (centre column) and 30 day aging (right column). Room temperature in light (top row), room temperature in the dark (second row), 4 OC (third row) and -20 OC (fourth row). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

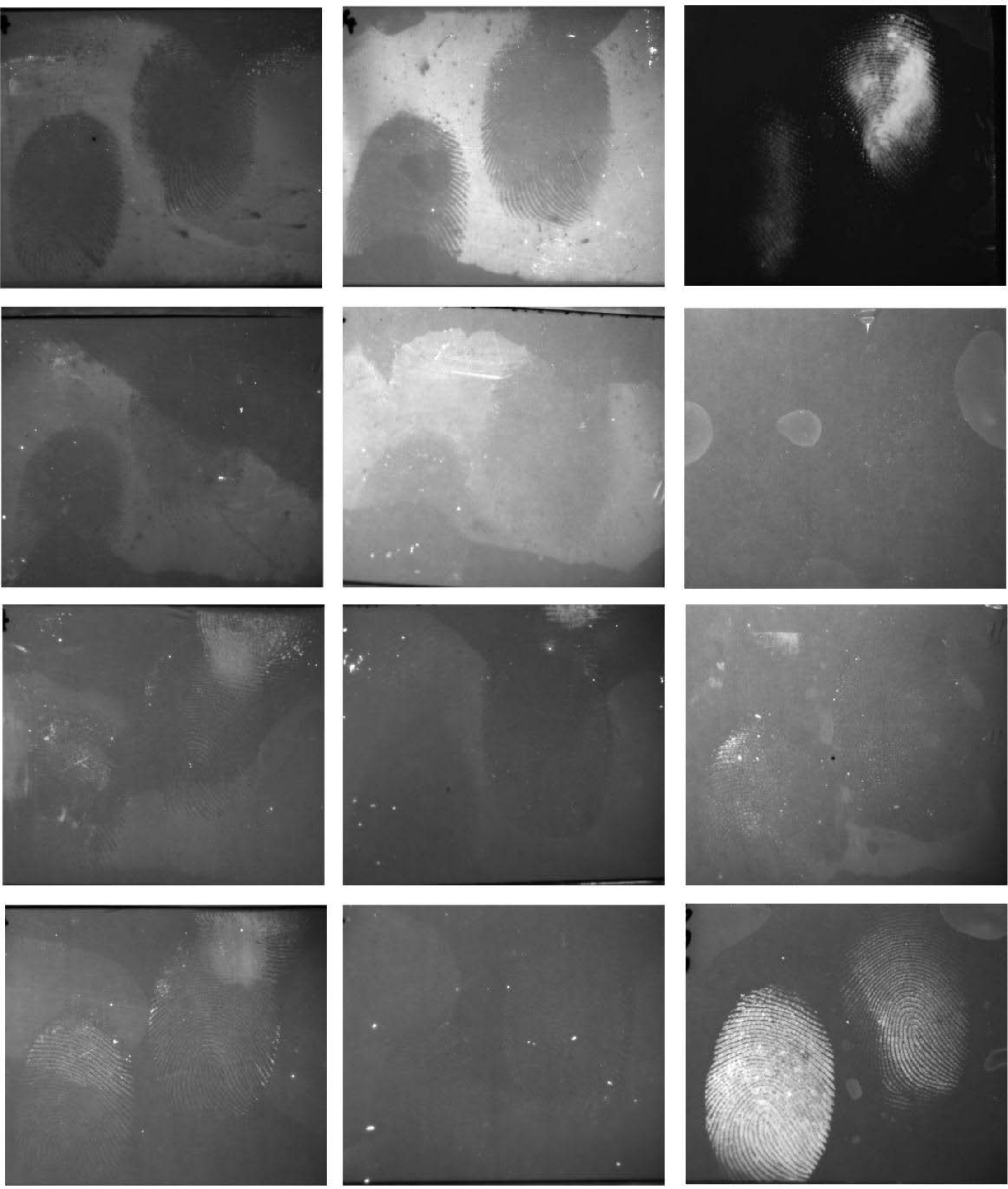

Figure A-2: Results from the effect of storage conditions with charged fingermarks from donor 3 developed with aptamer 1. Seven day aging (left column), 14 day aging (centre column) and 30 day aging (right column). Room temperature in light (top row), room temperature in the dark (second row), 4 ^{O}C (third row) and -20 ^{O}C (fourth row). Image recorded in the fluorescence mode using a 505 nm excitation light with a 555 nm band-pass camera filter.

Appendix II - Preparation and application of indanedione-zinc reagent.

Indanedione-Zinc reagent preparation and application as stated in the NCFS workshop manual [226].

Reagent preparation

IND stock solution (500 mL)

3 g	Indanedione, dissolved in
450 mL	Ethyl acetate, then add
50 mL	Acetic acid

Zinc chloride stock solution (200 mL)

8 g	Zinc chloride
200 mL	Absolute ethanol

IND-Zn working solution (100 mL)

100 mL	IND stock solution, diluted in
900 mL	HFE-7100 carrier solvent, then add
4 mL	Zinc chloride stock solution

Application

1. Singer magic steam press 7 preheated to 160 ^{O}C.
2. 200 ml of working solution placed into a small glass tray, kept in a fume cupboard.
3. Individual fingermark samples placed into developing solution for approximately 3 seconds before being removed and placed onto clean laboratory absorbent paper. Solution was discarded and replaced when the solution became cloudy.

4. Once all fingermark samples were treated and dried, they were placed into the heat press, sandwiched by laboratory absorbent paper, and heated for 10 seconds at 160 ^{O}C (no steam applied).

5. Fingermark samples were visualised using a Poliview PL 500 system using a band-pass filter of 505 nm with a camera barrier filter of 555 nm.

Appendix III – Publications and Conference proceedings arising from this work

Refereed publications

Wood, M., Maynard, P., Spindler, X., Lennard, C. & Roux, C., Visualization of Latent Fingermarks Using an Aptamer-Based Reagent, Angewandte Chemie International Edition, 51 (49) (2012) 12272-12274.

Wood, M., Maynard, P., Spindler, X., Lennard, C. & Roux, C., Selective targeting of fingermarks using immunogenic techniques, Australian Journal of Forensic Sciences 45 (2) (2013) (DOI 10.1080/00450618.2012.744847) (In press).

Conference proceedings

'A novel approach to latent fingermark detection using aptamer-based reagents'. 21st Australia and New Zealand Forensic Science Society International Symposium. Hobart. 24-27th September 2012.

'A novel approach to latent fingermark detection using aptamer-based reagents'. 1st Summer Doctoral School in Forensic Science and Criminology. Arolla. 27-29th August 2012.

'A novel approach to latent fingermark detection using aptamer-based reagents'. 6th European Academy of Forensic Science International Conference. The Hague. 20-24th August 2012.

'Protein detection in latent fingermarks using an aptamer-based reagent'. Australian Federal Police-University of Canberra-University of Technology, Sydney Forensic Research and Development Workshop. Sydney. 30th-31st July 2012.

'Novel amino-acid targeting immunogenic reagents for the detection of latent fingermarks on porous and non-porous substrates'. 19th International Academy of Forensic Science World Meeting. Funchal. 12-17th September 2011.

'Novel amino-acid targeting immunogenic reagents for the detection of latent fingermarks on porous and non-porous substrates'. International Fingerprint Research Group Meeting. 13-17th June 2011.

'Amino-acid detection in latent fingermarks using a novel aptamer-based reagent'. Australian Federal Police-University of Canberra-University of Technology, Sydney Forensic Research and Development Workshop. Canberra 27-28th July 2011.

'A novel approach to latent fingermark detection using aptamer-based reagents'. 20th Australia and New Zealand Forensic Science Society International Symposium. Sydney. 5-9th September 2010.