Preparing Ion Channel Switch Membrane-Based Biosensors

Amani Alghalayini[1,4,#], Charles G. Cranfield[1,4], Bruce A. Cornell[2,4], Stella M. Valenzuela[1, 3, 4]

[1]School of Life Sciences, University of Technology Sydney, Sydney, New South Wales 2007, Australia

[2]Surgical Diagnostics Pty Ltd., Roseville, Sydney 2069, Australia

[3]Institute for Biomedical Materials and Devices, University of Technology Sydney, Sydney, New South Wales 2007, Australia

[4]ARC Research Hub for Integrated Devices for End-user Analysis at Low-levels (IDEAL), Faculty of Science, University of Technology Sydney, NSW 2007, Australia

Running title: Membrane-based biosensor

[#]Corresponding author: Dr Amani Alghalayini

University of Technology Sydney,

Ultimo 2007, NSW, Australia

(TEL) +61 421 322 709

(e-mail) amani.alghalayini@uts.edu.au

SUMMARY

Monitoring the changes in membrane conductance using electrical impedance spectroscopy is the platform of membrane-based biosensors in order to detect a specific target molecule. These biosensors represent the amalgamation of an electrical conductor such as gold and a chemically tethered bilayer lipid membrane with specific incorporated ion channels such as gramicidin-A that is further functionalized with detector molecules of interest.

Keywords: Tethered Bilayer Lipid Membranes, Ion Channels, Gramicidin-A, Biosensor, Electrical Impedance Spectroscopy.

1. INTRODUCTION

A unique construction of ion biosensor switch technology, established by Cornell et al. in 1997 [1], has the potential to be used as a point of care diagnostic device to detect viruses, hormones, specific antigens and drugs [2,3]. A typical *ion channel switch* (ICS) membrane-based biosensor comprises self-assembled tethered bilayer lipid membranes (tBLMs) [4] that incorporates mobile gramicidin-A (gA) ion channels conjugated to antibodies via short linker molecules in the outer leaflet and anchored gA molecules in the inner leaflet of the membrane [5]. The ICS sensor mimics natural biological switches in order to provide label-free, real-time, high detection sensitivity measurements of analytes at very low concentrations by monitoring

transmembrane ion transport. The sensing platform measures the variation in membrane conduction due to the monomer-dimer reaction kinetics of gA.

There are two architectures of ICS biosensors, the *Direct ICS* assay and the *Competitive ICS* assay. Briefly, the Direct ICS biosensor is a powerful detection platform for detecting large analytes with multiple recognition sites, for instance, thyroid-stimulating hormone (TSH). The detection mechanism of the Direct ICS biosensor assay depends on trapping the introduced analyte by a mobile antibody fragment conjugated to a mobile gA monomer in the membrane outer leaflet and a stationary antibody fragment conjugated to a low-density stationary membrane spanner lipid. This would anchor mobile gA monomer distantly from a tethered gA monomer at the inner lipid membrane leaflet. As a result, the gA *dimer* conduction is prevented and analyte detection can be determined by a significant drop in membrane conduction, as illustrated in (Fig.1) [1].

On the other hand, the Competitive ICS biosensor is a powerful detection platform for detecting small analytes with only a single epitopic site, for instance, digoxin molecules. The detection mechanism of Competitive ICS biosensor assay depends on the competition between the analyte and previously incorporated molecules that mimic analyte on binding to antibody fragments, as illustrated in (Fig.2). Mobile gA monomers are kept immobilized in their monomeric form prior to introducing the analyte by trapping with molecules that mimic the analyte. Then the introduction of the sample of interest will compete with the previously attached analyte that mimics the antibody-binding site. This, in turn, will release the mobile

gA monomer permitting it to dimerize with their partner of immobile gA monomer within the inner leaflet of tBLM. Thus analyte detection can be determined by a significant *increase* in membrane conduction [1].

Here, we provide a protocol for the design of the Direct ICS biosensor and the Competitive ICS biosensor for targeted applications. The procedure covers the formation of the first and second layer of assembly onto freshly sputtered gold electrodes for both Direct and Competitive ICS biosensor configurations.

2. MATERIALS

Prepare and store all reagents at 4 °C temperature unless mentioned otherwise. The volumes and concentrations listed are appropriate for the 2.1 mm^2 gold electrodes fitted into an SDx Tethered Membrane Pty Ltd supplied 6 -well (6-electrode) cartridge (Note 1). Likewise, the instructions used for electrical impedance measures of the sensors are appropriate for a tethaPod™ AC electrical impedance spectrometer and tethaQuick™ software supplied by the same company. Concentrations and protocols may differ for other electrode and impedance spectroscopy platforms used.

1. Freshly sputtered gold electrode (Note 1).
2. Tethering Mixture of the Direct ICS biosensor (TMD solution). This consists of 50 µM benzyl disulfide-TEG-OH spacers, 7.5 nM tethered gramicidin-A (gAYYSSBn),

59 mM membrane-spanning phytanyl lipids (DLP), 4.4 mM full membrane-spanning lipids (MSL) and 4.4 nM biotinylated tethered membrane-spanning lipid (b-MSL) dissolved in the pure ethanol solution [1]. These compounds can be purchased solely through SDx Tethered Membranes Pty Ltd, Australia and their suppliers.

3. Tethering Mixture of the Competitive ICS biosensor (TMC solution). This consist of 150 µM benzyldisulfide-TEG-OH, 0.043 µM tethered gramicidin-A (gAYYSSBn), 300 µM membrane-spanning phytanyl lipids (DLP), 4.5 µM full membrane-spanning lipids (MSL) and 0.225 µM biotinylated tethered membrane-spanning lipid (b-MSL) dissolved in the pure ethanolic solution [6]. These compounds can be purchased solely through SDx Tethered Membranes Pty Ltd, Australia and their suppliers.
4. Mobile Components of the Direct ICS biosensor (MCD solution). This consists of 3 mM mobile phase lipids, comprising 70% zwitterionic C20 Diphytanyl-Glycero-Phosphatidylcholine lipid (DPEPC) and 30% C20 Diphytanyl-diglyceride-OH ether (GDPE) molar mixtures mixed with 350 nM biotinylated gramicidin-A (gA5XB) monomers. This mobile lipid solution is can be purchased solely through SDx Tethered Membranes Pty Ltd, Australia and their suppliers. The mobile components mixture is dissolved in 100% ethanol.
5. Mobile Components of the Competitive ICS biosensor (MCC solution) This comprises 3 mM mobile phase lipids of 70% zwitterionic C20 Diphytanyl-Glycero-Phosphatidylcholine lipid (DPEPC) and 30% C20 Diphytanyl-diglyceride-OH ether (GDPE) molar mixtures. The mobile lipid mixture is then mixed with 350 nM of the gA derivatized with a hapten (*see* Note 2). All mobile components are dissolved in pure

ethanol. These mobile lipid solutions can currently only be purchased solely through SDx Tethered Membranes Pty Ltd, Australia and their suppliers.

6. Biotinylated antibody fragments (B-Fab) in PBS buffer (*see* Note 3).
7. Phosphate Buffered Saline (PBS).
8. 1.6 µM streptavidin solution (stock solution) in PBS.

3. METHODS

3.1 Biosensor preparation

3.1.2 Formation of tethered components onto electrodes

1. Incubate freshly made gold slide in TMC or TMD solution, as desired, for 1 hour at room temperature with slight shaking (*see* Note 4).
2. Rinse the gold slide thoroughly by immersing in copious amounts of pure ethanol over 30 sec.
3. Use the gold slide with the tethered components directly for the next step or store it in a glass jar full of pure ethanol at 4 °C (*see* Note 5 and Note 7).

3.2 Preparation of mobile phase lipid bilayer

1. Using a micro-pipette, add 8 µl of the MCD or MCC solution corresponding to the desired biosensor type to the electrodes that have been pre-coated as per step 3.1 above (*see* Note 8 and Note 9). Incubate each electrode with these solutions for exactly 2 min at room temperature.

2. Rinse each electrode at least 3 times with 200 µL of PBS buffer. Always leave 200 µL of buffer over the electrodes in order to not expose the electrodes to air (*see* Note 10).

3.3 The Direct ICS biosensor preparation

1. Introduce 5 µl of the streptavidin solution, for a final concentration of 40 nM, to the liquid phase surrounding the membrane formed in 3.2 above.
2. Incubate streptavidin for 10 min at room temperature.
3. Rinse at least 3 times with 200 µl of PBS.
4. Add 20 µl of 0.2 µM b-Fab solution-of-interest to each well to get a 20 nM final concentration of b-Fab.
5. Incubate b-Fab for 10 min at room temperature.
6. Rinse at least 3 times with 200 µl of PBS.
7. Use the sensor for the next step or store in PBS solution at 4 °C until use (see Note 6 and Note 7).

3.4 The Competitive ICS biosensor preparation

1. Prepare mobile phase lipid bilayer with the same previously uses mixture that comprises gA - hapten and mobile lipids (70% DPEPC and 30% GDPE).
2. Introduce 5 µl of 1.6 µM streptavidin solution to the liquid phase above the membrane to get a 40 nM final concentration of streptavidin.
1. Incubate streptavidin for 10 min at room temperature. This will enable streptavidin to conjugate to the biotinylated full-membrane spanning tether molecules.
3. Rinse at least 3 times with 200 µl of PBS.

4. Add 5 µl of 1 µM b-Fab solution of interest to each well to get a 50 nM final concentration of b-Fab.
5. Incubate b-Fab for 10 min at room temperature in order to allow the conjugation between streptavidin and biotinylated-Fab.
6. Rinse at least 3 times with 200 µl of PBS. The sensor is now ready to use.

3.5 Sample preparation

Following the steps of assembling and preparation of the ICS biosensor, test 200 µl of the analyte of interest in 200 µl PBS buffer using electrical impedance spectroscopy. Always use one well for negative control by adding 200 µl PBS buffer only.

3.5.1 Measurement procedure

1. Insert prepared ICS sensor into a tethaPod™ AC electrical impedance spectrometer.
2. Using the tethaQuick™ software (SDx tethered Membranes Pty. Ltd., Australia), set up the hardware settings as follows: the settling time is 2 sec, the duration time is 4 sec, 25 mV peak-to-peak AC excitation time and continuous repeat interval. Set scanning frequencies from 1 Hz and 1 kHz, using four steps per decade.
3. Set an equivalent circuit model that describes the tethering gold electrode as a constant phase element in series with a resistor describing the electrolyte buffer and a parallel resistor-capacitor network to represent the lipid bilayer biosensor.
4. Start a real-time measurement of membrane capacitance (C_m) and membrane conduction (G_m) (*see* Note 11).

4. NOTES

1. For best results, polycarbonate microscope slides are very good for gold to adhere. Gold electrodes can be made by evaporating 100 nm, 99.9995% gold (5n5 gold) film onto these polycarbonate slides [7]. A polyethylene cartridge can then be attached that defines measuring wells. Cartridges that contain a counter electrode and pre-coated electrode slides can be purchased especially for these sensors through SDx Tethered Membranes Pty Ltd, Australia and their suppliers (Fig 3) [8].
2. For gramicidin-A derivatized with a hapten, hapten can be chemically conjugated to gramicidin-A, such as digoxin-gramicidin-A monomers.
3. The complementary antibody fragments B-Fab would couple to streptavidin intermediate conjugate that previously coupled to biotinylated mobile gA ion channels and biotinylated membrane-spanning lipids (b-MSL).
4. Use a freshly sputtered gold electrode to prepare the first monolayer in order to ensure that the slide surface is clean and has not had time to oxidize.
5. The prepared slides with the first monolayer can be stored at 4 °C for at least 6 months in ethanol.
6. The prepared slides with prepared ICS biosensor can be stored at 4 °C for at least 3 months in PBS.
7. It is essential to allow the slides and lipid solution to warm up to room temperature before starting the experiment to minimize measurement instability.
8. Be sure not to contact those gold-patterned areas where the tBLMs will form.

9. Do not let the edge of the micropipette tip touch the gold surface to avoid damaging the first layer of tethering chemistries.
10. It is important not to let the ICS sensors dry out once they have formed. As the ion switch components are embedded in a lipid bilayer, this bilayer will be irreparably damaged if the surrounding aqueous phase is removed. It is also important that no air bubbles are introduced between the tethering electrode and any return electrode, as this will affect any electrical impedance spectroscopy measurement.
11. Membrane capacitance values of typical ICS biosensors are 0.6±0.1 μF/cm^2 [6,9].

ACKNOWLEDGMENTS

This work was supported by the ARC Research Hub for Integrated Device for End-user Analysis at Low-levels (IDEAL) (IH150100028). We declare that Bruce Cornell is a shareholder of SDx Tethered Membranes Pty Ltd.

REFERENCES

1. Cornell BA, Braach-Maksvytis V, King L, Osman P (1997) A biosensor that uses ion-channel switches. Nature 387 (6633):580

2. Lee S-K, Cascão-Pereira LG, Sala RF, Holmes SP, Ryan KJ, Becker T (2005) Ion channel switch array: A biosensor for detecting multiple pathogens. Industrial Biotechnology 1 (1):26-31

3. Oh S, Cornell B, Smith D, Higgins G, Burrell C, Kok T (2008) Rapid detection of influenza A virus in clinical samples using an ion channel switch biosensor. Biosensors and Bioelectronics 23 (7):1161-1165

4. Alghalayini A, Garcia A, Berry T, Cranfield CG (2019) The Use of Tethered Bilayer Lipid Membranes to Identify the Mechanisms of Antimicrobial Peptide Interactions with Lipid Bilayers. Antibiotics 8 (1):12

5. Woodhouse G, King L, Wieczorek L, Osman P, Cornell B (1999) The ion channel switch biosensor. Journal of Molecular Recognition 12 (5):328-334

6. Woodhouse G, King L, Cornell B (1999) Kinetics of the competitive response of receptors immobilised to ion-channels which have been incorporated into a tethered bilayer. Faraday discussions 111:247-258

7. Moradi-Monfared S, Krishnamurthy V, Cornell B (2012) A molecular machine biosensor: construction, predictive models and experimental studies. Biosensors and Bioelectronics 34 (1):261-266

8. Cranfield C, Carne S, Martinac B, Cornell B (2015) The assembly and use of tethered bilayer lipid membranes (tBLMs). Methods in Membrane Lipids:45-53

9. Hoiles W, Krishnamurthy V, Cranfield CG, Cornell B (2014) An Engineered Membrane to Measure Electroporation: Effect of Tethers and Bioelectronic Interface. Biophysical journal 107 (6):1339-1351

Figure. 1 Schematic of the Direct ICS biosensor. ***A*** The gA ion channel is conductive due to the regular formation of dimers between mobile and immobile gA monomers in each membrane leaflet. ***B*** The mobile gA channel is unable to dimerize with its stationary gA monomer partner due to the coupling of mobile gA to the analyte. *C* Schematic representative of membrane conduction measurements overtime, whereas shifting from conductive dimers to non-conductive monomers (gated open to gated closed) and as a result, the overall measured membrane conduction will drop.

Figure. 2 Schematic of the Competitive ICS assay. ***A*** The mobile gA monomer is immobilized due to its interactions with molecules that mimic the sample. ***B*** The introduction of the analyte

will compete for the antibody-binding site, and as a result, it will release the mobile gA monomer, permitting it to dimerize with the immobile gA monomer in the adjacent membrane leaflet. ***C*** Schematic representative of membrane conduction measurements overtime, whereas shifting from non-conductive dimers to conductive monomers (gated closed to gated open) and as a result, the overall measured membrane conduction will rise.

Figure 3: Photographs of SDx electrode array. *A* Slide array with six working gold electrodes; *B* Assembled six-well flow cartridge that includes a counter electrode at the bottom of the cartridge.

Figure 1

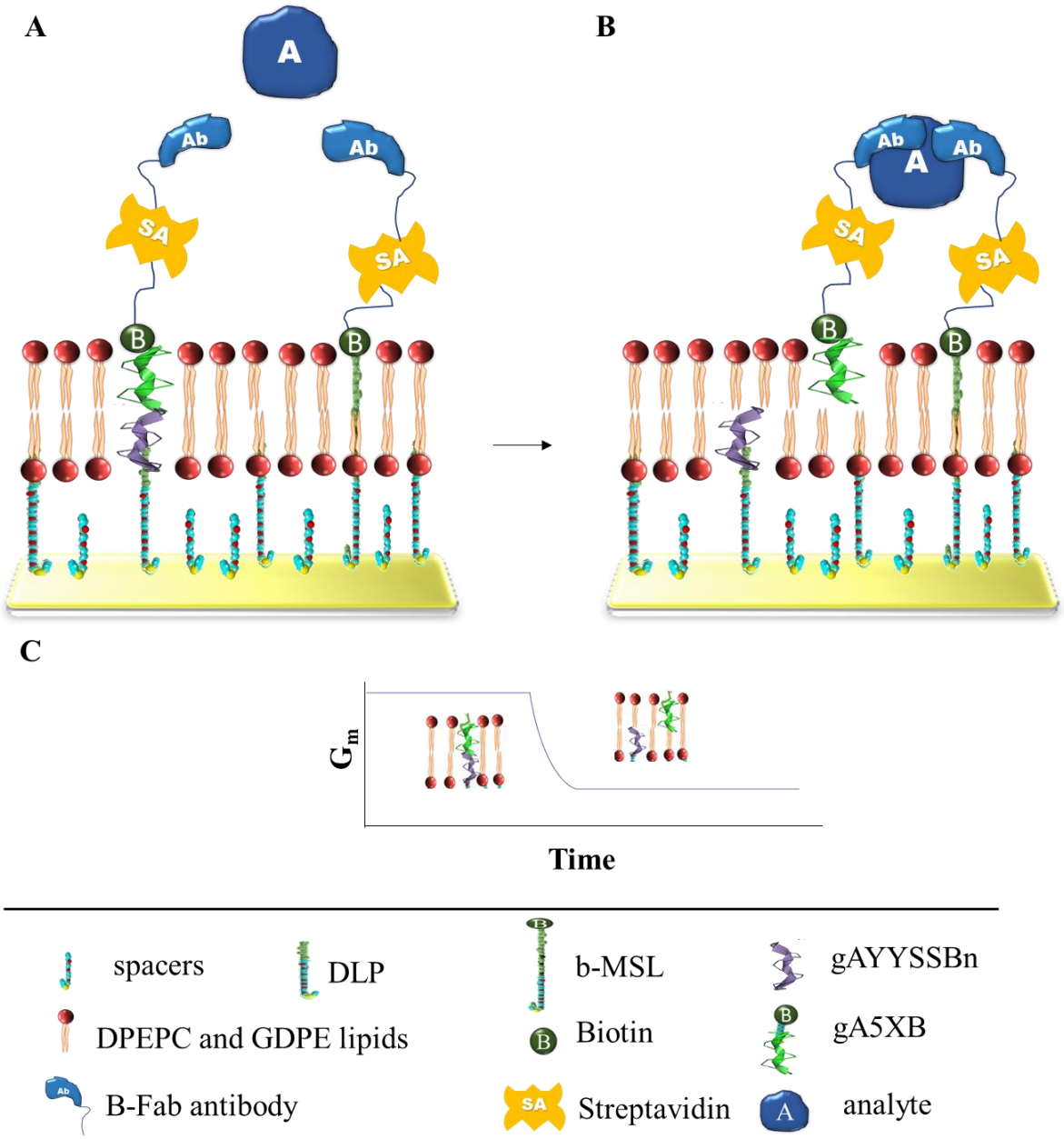
A
B
C
A
Ab
Ab
SA
SA
B
B
Ab
A
Ab
SA
SA
B
B
G_m
Time
spacers
DLP
b-MSL
gAYYSSBn
DPEPC and GDPE lipids
Biotin
gA5XB
B-Fab antibody
Streptavidin
analyte

Figure 2

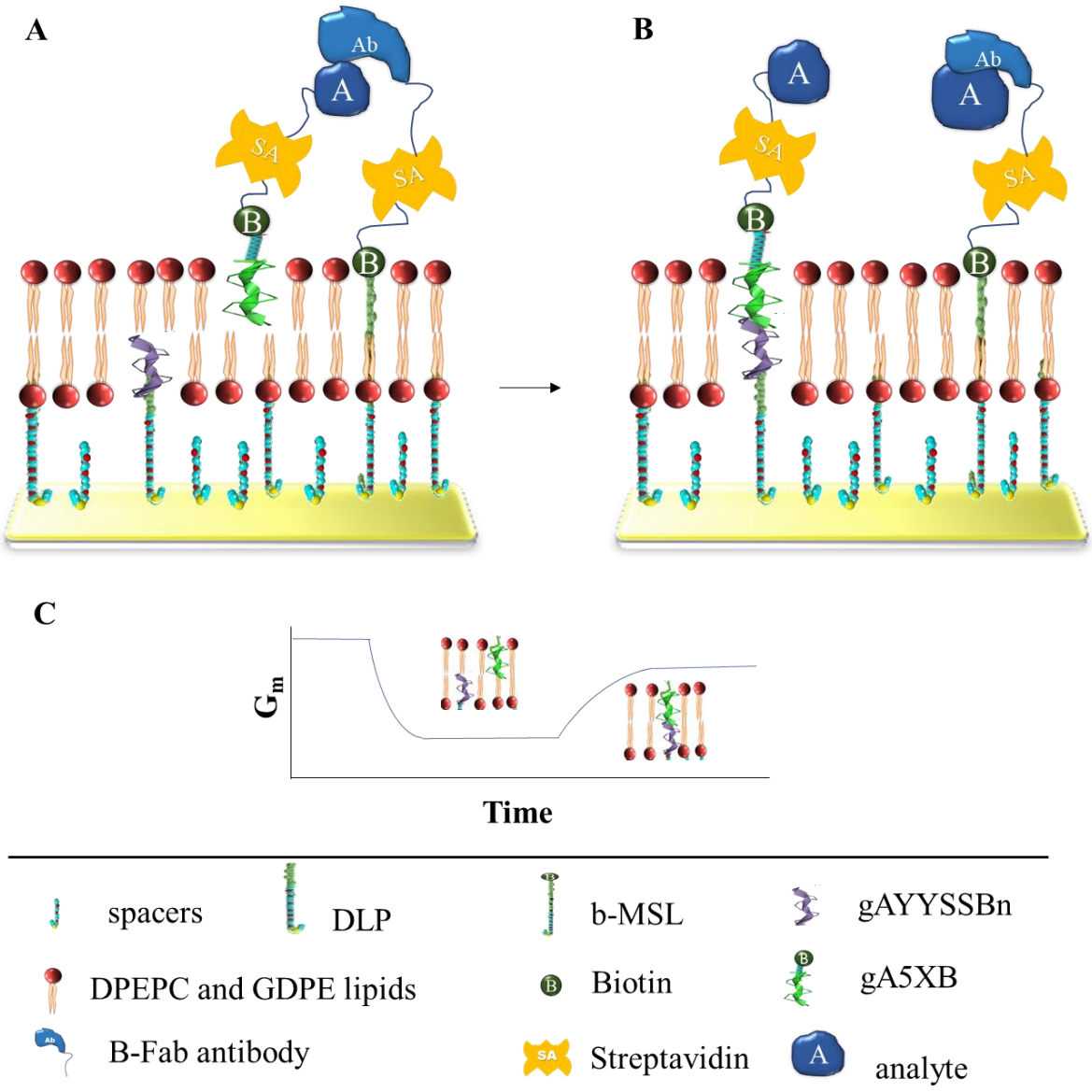
A
Ab
A
SA
SA
B
B
B
A
SA
B
Ab
A
SA
B
C
G_m
Time
spacers
DLP
b-MSL
gAYYSSBn
DPEPC and GDPE lipids
Biotin
gA5XB
B-Fab antibody
Streptavidin
analyte

Figure 3