Optical Detection of Integrin $\alpha_v\beta_3$ Using a Nanostructured Lipid Bilayer Membrane Biosensor

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ABSTRACT

An ultra-sensitive optical bionanosensor platform for integrin $\alpha_v\beta_3$ loaded human umbilical vein endothelial cells (HUVECs) was fabricated. The bionanosensor design involved the incorporation of an RGD (arginine-glycine-aspartate) containing peptide conjugated BODIPY lipid soluble dye (donor) into a substrate supported bilayer lipid membrane (SBLM). The RGD peptide binds with the integrin $\alpha_v\beta_3$ via a multimerisation mechanism thus reducing the energy transfer from the donor dye moiety to a second higher excitation wavelength BODIPY lipid soluble dye (acceptor) moiety. The reduction in energy transfer is visualised by an increase in the fluorescence intensity of the donor and a decrease in the fluorescence intensity of the acceptor. The surface chemistry of the substrate has a significant effect on the fluidity of the lipid bilayer (measured by Fluorescence Recovery after Photobleaching (FRAP)) and on the subsequent biosensing response. Using this novel bionanosensor architecture a sensitivity of 25 HUVEC cells/ml was recorded.

Keywords: Integrin, optical, bionanosensor, lipid membrane

1. INTRODUCTION

A novel approach to next generation biosensor applications is to mimic the natural lipid bilayer membrane. Due to the high hydrophilicity of these membranes the degree of protein non-specific adsorption is significantly reduced. The Swanson group (Song, 1999) have proposed the use of lipid bilayers incorporating fluorescent dyes as the signal transducer and Forster Resonance Energy Transfer (FRET) as the interrogation technique. This type of optical system is only partially affected by pinholes/non-uniformity of the lipid bilayer and is therefore more robust than electrical interrogation systems. In the Swanson biosensor the lipid bilayer is either directly attached to microbeads (Song, 2000) or a glass substrate for optical waveguide applications. (Kelly, 1999) Therefore transmembrane proteins such as the biological important acetylcholine molecule cannot be used in these biosensors because they will have a reduced function in the lipid membrane due to the close proximity of the substrate surface disrupting the 3D structure of the transmembrane protein. A number of methods have been investigated to enable the incorporation of transmembrane proteins into substrate supported bilayer lipid membranes, including the use of aqueous polymers (Wong, 1999), unsymmetrical SAMs (Jenkins, 1998) and/or tethered bilayers (Naumann, 2002) to act as bridging links between the substrate and the bilayer lipid membrane. Our research proposes the use of a biotin/streptavidin "cushion" linker between the substrate and the bilayer. Other groups (Berquand, 2003; Delrouye, 2002) are also investigating similar systems using the streptavidin/biotin link, but these methods require the use of fusogens (i.e. PEG) to trigger the vesicle fusion of the streptavidin bilayer. Previous work has demonstrated that by manipulating the vesicle lipid composition and the small unilamellar vesicle preparation, it is possible to form bilayer lipid membranes on streptavidin without the use of external reagents. (Worsfold, 2003a)

Integrin $\alpha_v\beta_3$ is a cell surface bound adhesion receptor that is important in cell-cell and cell-extracellular matrix (ECM) interactions. (Hynes, 1992) Integrins are heterodimers composed of α and β subunits that have been shown to bind with RGD containing peptide ligands possibly via the β_3 chain (D'Souza, 1994). The primary mechanism of the integrin signalling pathway is their ability to recognise ligands from the ECM, anchor, then multimerise on the cell surface. (Stupack, 2002) The arraying (multimerisation) of the integrin $\alpha_v\beta_3$ will form the basis of the present biosensor signalling transducer. [The receptor RGD peptide was covalently attached to a BODIPY fluorescently labelled lipid molecule to act as a donor chromophore.] In the presence of integrin, the RGD attached donor molecule will array on the sensor surface thus affecting the ability of the donor to influence the spectral characteristics of an acceptor labelled lipid soluble material also present in the supported bilayer lipid membrane. (Figure 1)

Our previous work using substrate supported fluid bilayer lipid membranes supported on a streptavidin "cushion" layer demonstrated a sensitivity to integrin $\alpha_v\beta_3$ loaded HUVEC's of 1000cells ml^{-1}. (Worsfold, 2003a) The present work utilises the same biosensing mechanism but with different fluorescent dyes working as donor:acceptors pairs. We also achieved an improved methodology for the isolation and purification of the RGD peptide-dye conjugate (donor) thus enabling a greater sensitivity towards Integrin $\alpha_v\beta_3$ loaded HUVECs. The influence of the substrate surface chemistry is also investigated in terms of lipid bilayer membrane fluidity, biosensing response characteristics and long-term stability of the biosensor.

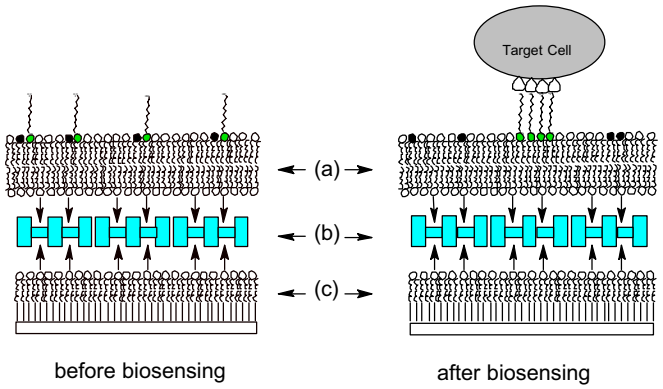

Figure 1: Schematic of the biosensor mechanism. (a)Lipid bilayer/RGDpeptide /BODIPY dye/Biotin lipid, (b) streptavidin, (c), EggPC/Biotin-PE LB monolayer. Substrate was HMDS treated glass or silicon.

2. EXPERIMENTAL

The materials used, synthetic methodology, substrate preparation and AFM characterisation, Langmuir monolayer studies and lipid bilayer membrane preparation will be described in full elsewhere (Worsfold, 2003b)

2.1 Lipid Bilayer Characterisation- FRAP

Fluorescence Recovery After Photobleaching (FRAP) measurements were performed using a Nikon ECLIPSE TS100-F microscope with an epi-fluorescent attachment. Suitable filters were used to allow broad-band excitation from 500-530nm and emission above 550nm. The sample was placed between two hematocytometer plate holders with a kalrez o-ring between the two plates. Photobleaching of the sample (r = 50μm at x20 magnification) with white light for 15-30secs was dependent on the sample. Images were recorded using a Hamamatsu C4742-95 digital camera with exposure times of 122ms – 1000ms depending on the sample. The images were analysed using SCION image beta 4.02 freeware.

2.2 Integrin $\alpha_v\beta_3$ Loaded HUVEC Biosensing

Fluorescence spectral analysis was performed using a Perkin-Elmer LS55 luminescence spectrometer. Scan speed = 25nm min^{-1}, Excitation slit = 2.5nm, Emission slit = 15nm. Excitation λ = 545nm (unless stated). The lipid bilayer samples were assembled/fabricated then inserted into a Perkin-Elmer cuvette slide holder. Biosensing experiments were performed by removing aliquots of the PBS solution and adding equal volumes of the HUVEC solution to the cuvette. The sensitivity of the biosensing response is determined from the following equation.

$$S = \frac{|F_0 - F_t|}{F_0} \times 100 \qquad (1)$$

Where S = Sensitivity of biosensing response, F_0 = Fluorescence (at a given wavelength) before and F_t = Fluorescence (at a given wavelength) after addition of cells respectively.

3. RESULTS AND DISCUSSION

The Langmuir monolayer and Langmuir-Blodgett film deposition characterisation are described elsewhere. (Worsfold, 2003b)

3.1 Fluorescence Recovery After Photobleaching (FRAP)

The fluidity of the lipid bilayer is described by the diffusion of fluorescently tagged lipids into an area, which was previously photobleached. The diffusion coefficient can be estimated from the following equation.

$$D = \left(\frac{r^2}{4t_{50}} \right) \qquad (2)$$

Where D is the diffusion coefficient, r = radius of the photobleached area and t_{50} = time taken to 50% fluorescent intensity recovery.

The lipid membrane fluidity of the ruptured vesicles was measured on three different surfaces (a) NaOH treated glass, (b) HMDS/streptavidin treated glass and (c) untreated glass. Figure 2 shows characteristic fluorescent micrographs produced in these FRAP studies for a lipid bilayer formed on a NaOH treated glass substrate. The t_{50} time is calculated from the fractional fluorescence recovery curve shown in figure 3 which gives a diffusion coefficient for this system D = $8.3 \times 10^{-8} cm^2 s^{-1}$. Also shown in figure 3 are the fractional fluorescence recovery curves for the untreated glass substrates supported lipid bilayer and the HMDS/streptavidin supported lipid bilayer. The diffusion coefficients for the HMDS/streptavidin supported lipid bilayer (D = $2.6 \times 10^{-8} cm^2 s^{-1}$) and the untreated glass substrate (D = $3.1 \times 10^{-8} cm^2 s^{-1}$) indicate that the fluidity of the lipid bilayer on NaOH treated glass is greater than on either the untreated surface or the HMDS treated/streptavidin "cushion" substrates both of which have similar values. However, the long term stability of the lipid bilayer on NaOH treated glass is significantly less than on the other substrate surfaces.

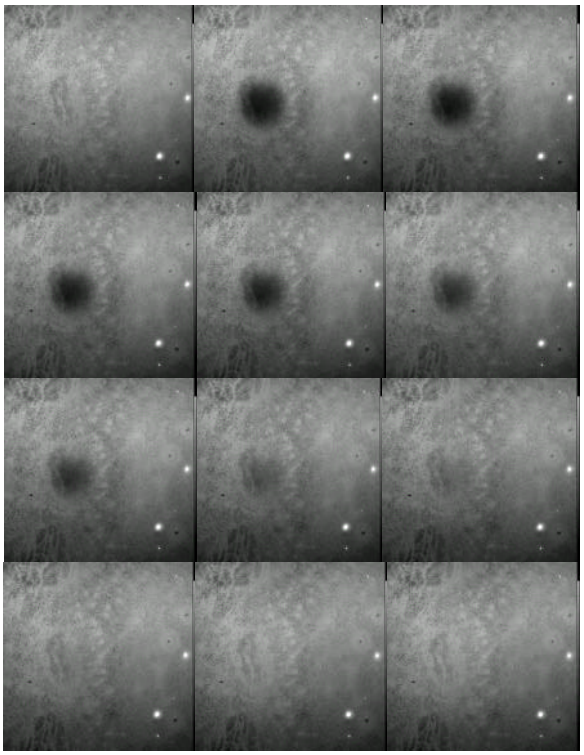

Figure 2: FRAP images of a lipid bilayer membrane on a NaOH treated glass substrate. From top left to right :- before photobleaching, after 1min photobleaching, 1min after photobleaching, 2mins after, 3mins after. 4mins after, 5mins after, 10mins after, 14mins after, 20mins after, 45mins after, 60mins after.

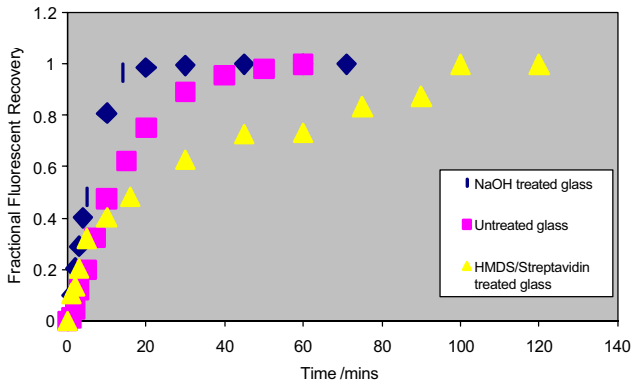

Figure 3. Fractional fluorescence recovery for lipid bilayer membranes on NaOH treated glass, untreated glass and HMDS/Streptavidin treated glass substrates.

3.2 Biosensing Experiments

All biosensing experiments were recorded on glass substrates etched for 24hrs, HMDS treated substrates and untreated substrates, at 298K. Figure 4 shows the effect of adding HUVEC cells, at a concentration of 25cells ml^{-1}, to a lipid bilayer formed on NaOH treated glass substrates. Here, there is an increase in the intensity of the BODIPY 558/568-peptide donor moiety at 570nm and a decrease in the intensity of the BODIPY TR-X acceptor moiety at 625nm on exposure to HUVECs. These changes in the fluorescence are predicted from the detection mechanism, where the donor peptide moieties multimerise on the lipid membrane surface upon binding with the integrin $\alpha_v\beta_3$ proteins on the HUVEC surface. This causes a reduction of the energy transfer from the donor to the acceptor moiety. Figure 5 shows the effect of 5000 HUVECcells ml^{-1} on the streptavidin "cushion" lipid bilayer structure. A lower degree of response is observed for this system compared with the NaOH treated glass system at a concentration of 25 HUVECcells ml^{-1}.

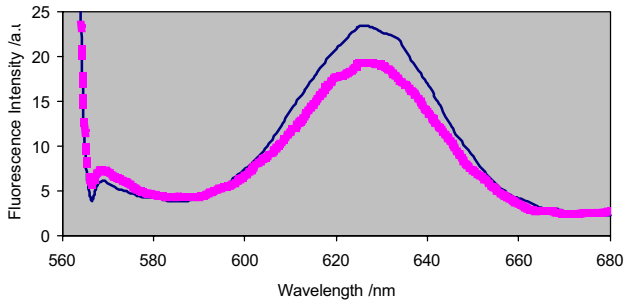

Figure 4. Effect of 25 HUVEC cells ml^{-1} (Thick line) to lipid bilayer membrane on NaOH treated glass substrate.

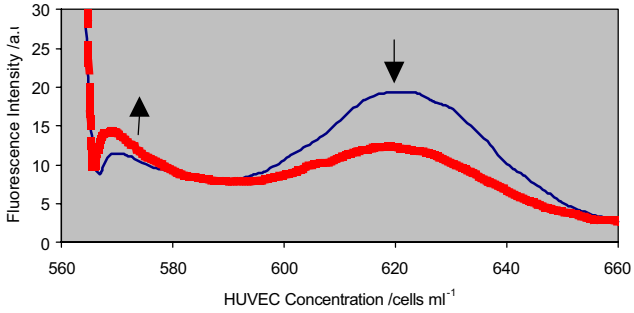

Figure 5. Effect of 5000 HUVECcells ml^{-1} (Thick line) to lipid bilayer membrane on HMDS/streptavidin treated glass substrate.

The effect of concentration on the sensor responses for both systems is shown in figure 6. In the lipid bilayer on NaOH treated glass substrate system (squares), there is a rapid increase in the response at low concentrations 0-500cells ml^{-1} followed by the onset of equilibrium saturation above this concentration. A lower degree of response is observed for the HMDS/streptavidin system compared with the NaOH treated glass system at a HUVEC concentration of 25cells ml^{-1}. However, the concentration plot of the HMDS/streptavidin supported system has a potentially greater dynamic range than the NaOH treated glass system. The long-term stability of the NaOH treated glass system is reduced (approx. 1 week) compared with the streptavidin "cushion" lipid structure which has demonstrated biosensing action after being stored

for 1 month at 4°C. The untreated glass substrate bilayer system also exhibited response on exposure to HUVECs but at a significantly lower sensitivity.

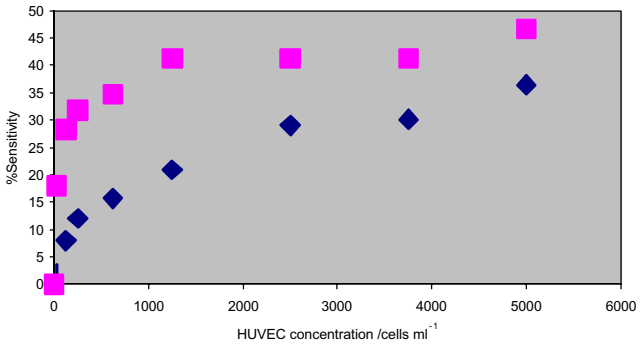

Figure 6. Effect of HUVEC concentration on response sensitivity for lipid bilayers on NaOH treated glass substrate (squares) and HMDS/streptavidin treated glass substra (triangles).

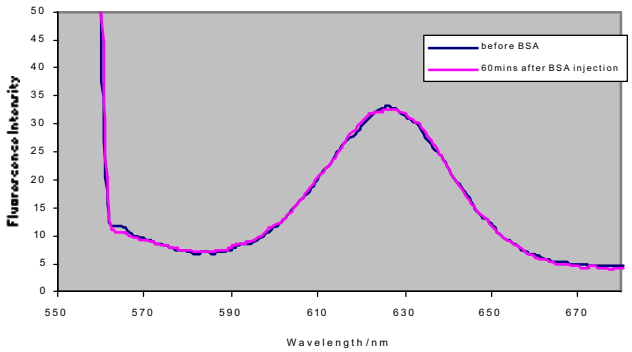

Figure 7. Effect of 63µm Bovine Serum Albumin on biosensor response.

Figure 7 shows the lack of any response of the HMDS/streptavidin system on exposure to 63µM Bovine Serum Albumin (BSA). This demonstrates the specificity of the proposed biosensor even on exposure to common interference proteins (BSA), which often cause false positive results in other optical biosensor systems.

4. CONCLUSIONS

In the present work we have changed the donor and acceptor dyes from the previous study to enable higher wavelength biosensor detection. Using this strategy, together with alterations in the donor:acceptor dye ratio, a higher sensitivity of 25 HUVEC cells ml^{-1} was recorded. The importance of the substrate surface chemistry was highlighted by the increased bilayer fluidity on the NaOH treated substrates. One possible reason for this is the increased flatness of these substrates compared with the HMDS/streptavidin, or untreated glass substrate system. Interestingly however, the HMDS/streptavidin system provided the greatest long-term stability, as well as good biosensing sensitivity and dynamic sensing range.

5. REFERENCES

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