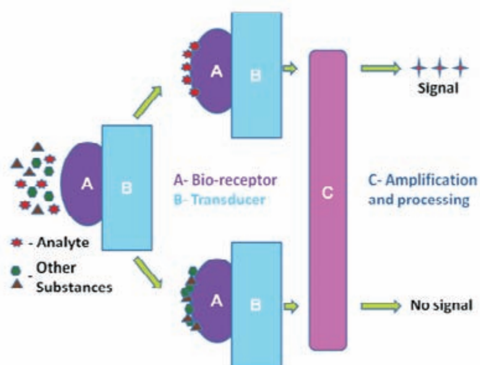
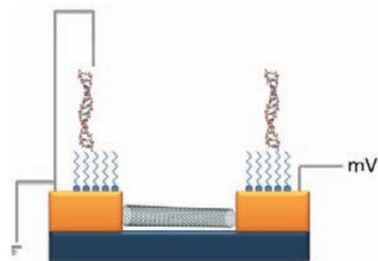
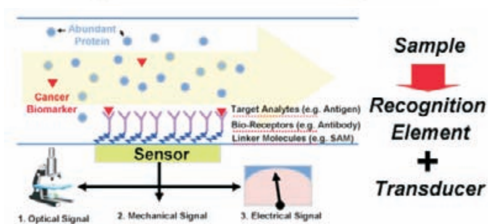
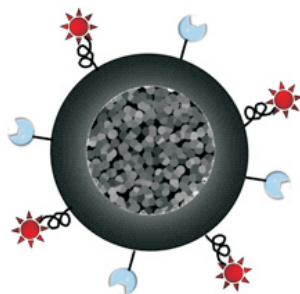
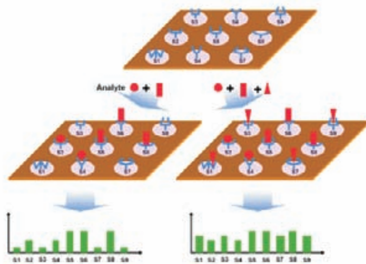
Biosensors and Cancer











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A SCIENCE PUBLISHERS BOOK

Biosensors and Cancer

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Biosensors and Cancer

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Preface

Biosensors have a simplistic concept but a great deal of sophistication in design, manufacture and application. They essentially have biological components within them and are used to detect, monitor or quantify substances. They use a variety of physical platforms and technologies. The biological components may include enzymes, membranes and cells or any other naturally occurring biological product. Some have artificial biological components such as modified molecules or polymers. Biosensors may be used to detect single or groups of molecules and have wide applicability to the life sciences. Each chapter in **Biosensors and Cancer** has an abstract, key facts, applications to other areas of health and disease and a "mini-dictionary" of key terms and phrases within each chapter. Finally, each chapter has a series of summary points. In this book focussing on cancer we have chapters on biosensors based on or utilizing optical imaging, surface plasmon resonance, microcantilevers, electrochemistry, aptamers, fluorescence, electrochemistry, nanobiosensors and nanowires. There are also chapters on oxidative damage to DNA, miRNA, leukemia, breast cancer, BCR-ABL activity, single living cells and thyroid cancer. Drug discovery, cancer diagnosis, anticancer drugs, and cancer detection identifying marker molecules for prostate cancer are also covered. Contributors to **Biosensors and Cancer** are all either international or national experts, leading authorities or are carrying out ground breaking and innovative work on their subject. The book is essential reading for oncologists, cancer workers and scientists, medical doctors, health care professionals, pathologists, biologists, biochemists, chemists and physicists, general practitioners as well as those interested in disease and sciences in general.

The Editors

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SECTION 1: GENERAL

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1

Functional Optical Imaging-based Biosensors

Pablo Iglesias[1,a] and Jose A. Costoya[1,b,*]

ABSTRACT

In vivo fluorescence and bioluminescence imaging are the most common techniques used in optical imaging. These methods, especially those based on fluorescent light emission, are provided with a high number of fluorescent tracers, either small organic dyes such as ICG or fluorescent proteins as in the case of GFP. Although tissue autofluorescence seems to be a major drawback of the technique when applied to imaging in living animals, the development of novel tracers able to emit in near-infrared wavelengths has given a boost to this method. On the other hand, bioluminescent light is emitted upon the chemical reaction of the enzyme luciferase with its substrate producing an emission peak between 560 and 580 nm. Both of these techniques have been commonly employed in cell biology as reporter assays that usually apprise on cell compartmentalization of gene products, transcriptional activity of interest genes or pathophysiological processes of diseases such as cancer. In the case of cancer, the transcription factor HIF-1α is a major regulator of the cell response to hypoxia by eliciting formation of neovessels. With all of these in mind, we have designed and characterized a BRET-based genetically encoded biosensor able to detect variations in the concentration of HIF-1α. Here we review our work in the field along

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List of abbreviations after the text.

with some remarkable examples of the application of optical imaging, either fluorescence and bioluminescence or BRET, to the development of biosensors.

INTRODUCTION

Classes of Imaging Methods

Traditionally, imaging methods applied to medicine would be separated in two broad categories, anatomical and functional imaging. Thus, anatomical imaging makes use of tissue contrast differences to locate affected organs, bones or detect tumours when applied to oncology. In this category, the most popular examples are magnetic resonance imaging (MRI) and X-ray computed tomography (CT), which have been successfully established as reference techniques in the clinic, playing a prominent role in the last decades since their debut as diagnostic tools. However, the limitations of these techniques are their inability to give any relevant information other than size or location of the pathophysiological features of the disease (Seaman et al. 2010). Unlike these, a second category comprises of imaging methods that are able to give more precise and detailed information about physiological processes such as oxygenation rate, perfusion and alterations of blood flow. In this regard, a variation of the MRI technique, functional MRI (*f*MRI) is currently being employed as a non-invasive method to preoperatively map functional cerebral cortex and to identify eloquent areas of the cerebral cortex in relation to brain cancers (Torigian et al. 2007). A third category could be added to these two traditional categories, molecular imaging methods, which are rapidly gaining popularity due to the better understanding of the molecular bases of disease. Formally, they can be defined as "the visualization, characterization, and measurement of biological processes at the molecular and cellular levels in humans and other living systems" (Mankoff 2007), i.e. these techniques inform on the molecular mechanisms underlying the biological processes of interest (Dunn et al. 2001; Alavi et al. 2004; Torigian et al. 2007).

Molecular Imaging: Optical Methods Overview

However, some of these techniques rely on ionizing radiation, such as CT and SPECT, involving higher doses than common X-ray imaging procedures, increasing exposure to radiation in the population that might be considered a public health issue in the future (Brenner 2007). And while the resolution of MRI and its ability to confer anatomic detail are difficult to match, it also requires extremely expensive instrumentation and is time-consuming, giving a poor throughput performance. On the other hand, optical methods

such as fluorescence and bioluminescence rely on the emission of visible light that, unlike the former ones do not display the harming effects of ionizing radiations on living organisms or require high-budget equipment to monitor the overall process (Sampath et al. 2008).

Fluorescence has risen to a distinguished position in molecular and cell biology thanks to the widespread use of the *Aequoerea victoria* green fluorescent protein (GFP) that as of today remains one of the most common fluorescent reporters. Since then, it has developed a whole array of new fluorescent proteins with diverse excitation and emission wavelengths that comprises almost the whole visible spectrum, making this technique very versatile for a wide range of applications (Shaner et al. 2005). In addition, these novel fluorescent proteins display interesting features such as NIR-shifted emission wavelengths that permit avoiding overlapping emissions from tissue and/or organic compounds (Ntziachristos et al. 2003; Weissleder and Ntziachristos 2003).

In bioluminescence, luciferase enzymes are commonly employed as reporter genes in cell and molecular biology. Sources of luciferases are insects such as the firefly *Photynus pyralis*, marine invertebrates such as the sea pansy (*Renilla reniformis*), plants (*Gaussia princeps*) and several species of vibrionaceae (Hastings 1983). Unlike fluorescence where electron excitation and subsequent photon emission is mediated by a physical phenomenon (light absorption), bioluminescence light is chemically produced by decaying singlet state species that emit photons of visible light. These "light-emitting" reactions are catalyzed by luciferase enzymes when their chemical substrates such as D-luciferin or coelenterazin are present and subsequently oxidized. Although fluorescent light is usually brighter, with less light scattering and photon attenuation that makes fluorescence more suitable for 3D reconstruction, bioluminescent light lacks the problem of cell and tissue auto-luminescence, fluorescent photobleaching and in general facilitates quantitative imaging in deeper localizations than fluorescence. Besides, the substrate D-luciferin becomes readily available upon administration, able to cross the blood-brain barrier upon intraperitoneal or intravenous administration and lacking any harmful effect on living organisms when regularly administered (Edinger et al. 2002).

Bioluminescence Resonant Energy Transfer (BRET)

Transference of resonant energy is a well-known phenomenon on which proteomic and biochemical procedures rely on to determine protein-protein interactions (Pfleger and Eidne 2006). In a similar way as FRET where a donor fluorochrome is able to excite a second fluorochrome acting as an acceptor, in BRET a luminescent donor (Luc) excites a fluorochrome. As Fig. 1.1 shows, when the FLuc substrate, D-luciferin is present this is

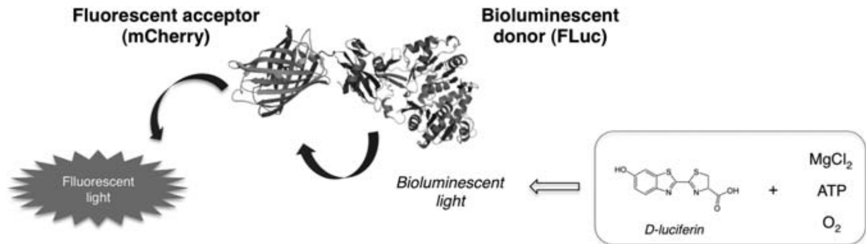

Figure 1.1. Outline of BRET occurring in a fusion protein where FLuc is the bioluminescence donor and a NIR fluorochrome is the fluorescence acceptor. The luciferase requires the presence of its substrate D-luciferin plus ATP, magnesium chloride and ATP to emit light. This light when close enough is able to excite the fluorochrome when the signal is registered.

oxidized by the luciferase emitting light as a by-product. This light is emitted high enough to excite a red fluorescent protein such as mCherry, a derivative of DsRed from *Discosoma* sp. (Shaner et al. 2004).

The efficiency of energy transfer is strongly dependent on Förster distance (R_0), the distance between the donor and acceptor generating 50% of the maximum possible energy transfer, which typically falls into the range of 1–10 nm. The orientation and freedom of movement of both proteins, in the case of fused pairs, can also be empirically tuned by inserting flexible linkers in between both proteins of appropriate length (Michelini et al. 2004; Dacres et al. 2010; Prinz et al. 2006). Although the choice of the suitable donor/acceptor pair is usually determined empirically, one of the most popular pairs is RLuc/GFP since this pair exhibits a spectral overlap of donor emission and acceptor excitation, which is one of the critical steps on the overall performance of the system.

Examples of Optical Methods Applied to Functional Imaging Biosensors

To illustrate the application of optical imaging methods to functional imaging biosensors we chose several outstanding examples. Bioluminescent imaging has been largely employed as transcription reporter *in vitro* assays by fusing response elements of target transcription factors to luciferase genes. These constructs may also be used to monitor the formation of grafted tumours *in vivo* and measure cell numbers during tumour progression and response to therapy. One of the best examples of applications of this type of biosensors is the E2F1-Luc transgenic mouse (Uhrbom et al. 2004). In this study, the authors report the construction of a chimeric construct formed by several response elements for E2F1, a master regulator of cell cycle progression that appears up-regulated in a high proportion of tumours, fused to a FLuc gene. Upon generating the transgenic mouse harbouring this biosensor they generated brain tumours by injecting intracranially

DF-1 cells, in order to recreate a PDGF-driven glioma model. Once the tumours were established and detected by BLI output, they submitted those mice affected with bioluminescent tumours with PTK787/ZK222584, an inhibitor of PDGF receptor and the rapamycin analog CCI-779. This treatment resulted in reduced light production that evidenced inhibition of cell proliferation of the tumour masses.

A similar strategy involves the use of tissue specific promoters coupled to a reporter gene. Bhang and colleagues (Bhang et al. 2011) report that the progression elevated gene-3 (PEG-3) promoter can be used to detect micrometastases in mice models of human melanoma and breast cancer either by BLI or employing radionuclides-based imaging techniques. The authors injected cells from either breast or human melanoma cells and then the pPEG-Luc (BLI) and pPEG-HSV1tk (PET) constructs along with linear polyethylenimine (l-PEI) polyplexes, as a means of gene delivery. Several masses were successfully detected and upon histological examination identified as micrometastasis. The accuracy of localization and size of these tumoural masses was further confirmed by whole-body acquisitions of SPECT-CT.

BRET may also be used as a basis of a biosensor as the self-illuminating quantum dots conjugated with luciferase reported by So and colleagues (So et al. 2006). Quantum dots are semiconductor nanocrystals with different optical properties depending on its size and its composition (Medintz et al. 2005). In this case, the authors conjugated several copies of RLuc8, an engineered red-shifted variant of *R. reniformis* luciferase designed to excite several polymer-coated ZnS/CdSe core shell quantum dots that emit fluorescent light at 655, 705 and 800 nm. Also in order to increase the cell uptake of these nanostructures, these functionalized quantum dots were conjugated with a polycationic peptide. The system proved to be highly effective in all cases (655, 705 and 800 nm) producing a quantifiable BRET signal thus demonstrating the possibilities of BRET-based biosensors modulated by specific biological interactions.

Applications to Areas of Health and Disease

These techniques are expected to fully develop in the forthcoming years as a prognostic tool for the clinical environment and more specifically for cancer treatment. In this context, to minimize the risk of recurrence it is desirable that the entire tumour is removed before metastasis takes place. In this regard, several cancer hallmarks can been used to design targeted molecular probes such as increased growth (augmented production of growth factor and growth factor receptors), unrestricted replicative potential, sustained angiogenesis and invasiveness of neighbouring tissues and/or metastatic abilities (Keereweer et al. 2010). One good example of

this approach are the activatable probes developed by Weissleder and colleagues (Wunderbaldinger et al. 2003; Mahmood et al. 2003) and currently commercialized by VisEn Medical (ProSense and AngioSense), which target metalloproteinases abundant in the surroundings of the tumour. These probes are administered in a quenched state, only displaying a basal emission until cleaved by its target protease.

The first steps for translation of this technique to the clinic have been made in sentinel lymph node mapping. The presence of cancer cells in regional lymph nodes indicates metastasis and necessitates more aggressive, systemic treatment, such as chemotherapy. In this regard, several studies report the use of intraoperative near-infrared fluorescence monitoring employing low weight molecular ligands (peptides and small molecules) able to target tumoural cells in their niches. This approach is currently being assessed as prospective intraoperatory assistance to surgeons (Soltesz et al. 2006; Tanaka et al. 2006) with NIR-emitting derivatives of indocyanine green (ICG).

Bioluminescence Resonance Energy Transfer (BRET)-based Biosensors and the Transcription Factor HIF-1alpha

In this chapter, we describe the design, construction and characterization of a novel hypoxia genetic biosensor with near-infrared fluorescence (NIR-F) and bioluminescent properties. This genetic biosensor comprises a regulatory moiety activated by the hypoxia inducible factor HIF-1α, enabling the transcription of a fusion protein that acts as a dual fluorescence-bioluminescence tracer capable of BRET-mediated fluorochrome excitation. All of these data and the corresponding materials and methods employed have been previously described (Iglesias and Costoya 2009).

Hypoxia as a Tumoural Aggresivity Marker in Cancer

One of the most recognizable features of a tumoural cell is the chaotic growth that is intimately related to tumour aggressiveness and invasiveness. As normal cells do, these tumoural cells secrete angiogenic signals to attract additional blood supplies as a response to hypoxia, which usually ensues as soon as the tumour enlarges beyond a millimetre or two in diameter. As a consequence of the low levels of oxygen (hypoxia), the angiogenic switch of these tumoural cells is activated resulting in secretion of hypoxic transcription factors and the number of blood vessels supporting the tumour rises exponentially to fulfil the exacerbated need of nutrients and oxygen (Alberts et al. 2009). This angiogenic process is tightly regulated and results in the participation of several transcription factors, with HIF-1α

being one of the most important. HIF-1 is a HIF-1α/HIF-1β heterodimer that binds the hypoxia response elements (HREs) of target genes under hypoxic conditions. The HIF-1β subunit is constitutively expressed, while in the case of HIF-1 subunit its expression and transcriptional activity are precisely regulated by the cellular O_2 concentration (Wang et al. 1995). As shown in Fig. 1.2 , by interacting with the coactivator CBP/p300, HIF-1 activates transcription of target genes involved in glucose transportation and glycolysis, angiogenesis, survival and proliferation, and invasion and metastasis (Bárdos and Ashcroft, 2004). In fact, HIF-1 is overexpressed in may cancer types and is associated with poor prognosis and its expression correlates with metastatic potential of those tumours (Semenza et al. 2003; Zhong et al. 1999).

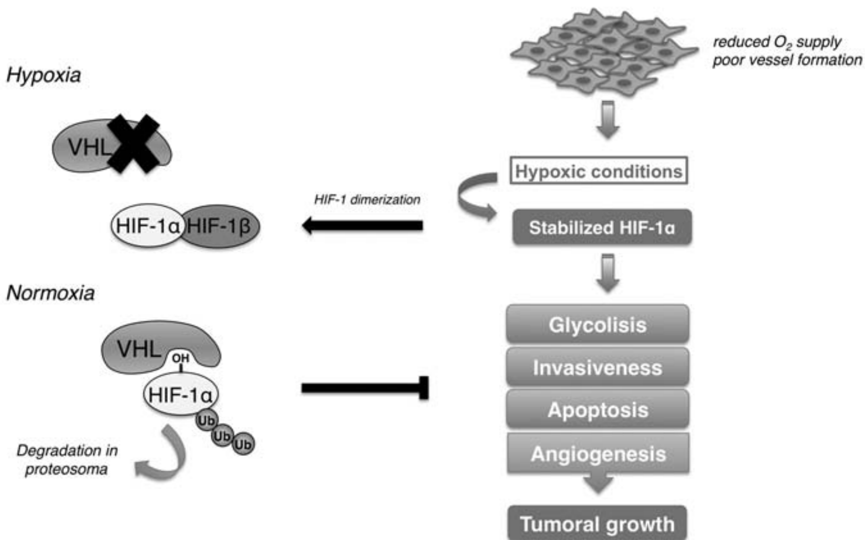

Figure 1.2. In normoxic conditions where oxygen is available the transcription factor HIF-1α becomes hydroxylated and subsequently poly-ubiquitined by the E3 ubiquitin ligase pVHL. On the other hand, in hypoxic conditions such as those within tumoural masses, the formation of neovessels and therefore oxygen supply is deficient and HIF-1α is stabilized and dimerizes with HIF-1β translocating to the nucleus and exerting its transcriptional activity on several essential processes for tumour development.

Design and Characterization of the Biosensor

Figure 1.3 shows the scheme of the biosensor. The HIF-1 sensor is formed by a novel chimeric enhancer able to bind the HIF-1α transcription factor more efficiently than the canonical hypoxia response elements (HRE). This chimeric enhancer that comprises a (Egr-1)-binding site (EBS) from the Egr-1 gene, a metal-response element (MRE) from the metallothionein

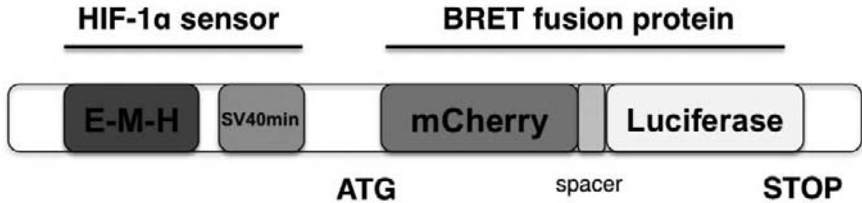

Figure 1.3. Schematic outline of genetically encoded biosensor, the E-M-H enhancer (EGR/MGR/3xHRE) and the SV40 minimal promoter comprise the HIF-1α sensor moiety, while the tracer module is formed by the fusion protein of mCherry and the firefly luciferase (FLuc).

gene, and a triplet of hypoxia-response elements (3xHRE) from the phosphoglycerate kinase 1 gene (Lee et al. 2006). A SV40 minimal promoter is located downstream of the chimeric enhancer E-M-H. The specificity of this biosensor for HIF-1α is of great advantage when compared to other angiogenesis biosensors based on promoter regions of other transcription factors such as VEGF (Salnikow et al. 2002). VEGF regulates and is regulated by numerous physiological and pathological processes, making a high number of cytokines and growth factors signalling pathways that are known to modulate VEGF expression at the transcriptional level. Some examples of this are IL-lβ and IL-6 cytokines, PDGF-BB, TGF-β, and transcription factors such as basic fibroblast growth factor, EGF and HGF, which makes this promoter too promiscuous to be taken solely as a reliable marker of hypoxia (Akagi et al. 1998).

On the other hand, the dual tracer moiety is formed by a fusion protein of mCherry and the firefly luciferase (FLuc). Although initially mPlum was tested as a prospective fluorochrome, it was eventually discarded due to its low brightness (data not shown and Shaner et al. 2005). On the other hand, mCherry presents an excitation wavelength of 585 nm, which makes this fluorochrome an ideal acceptor of bioluminescent light (575 nm), as well as its near NIR-emission avoids the autofluorescence phenomena occurring in living tissue.

At first, we wanted to assess the fluorescent and bioluminescent activity of the fusion protein in order to disregard any sequence discrepancy with the previously reported excitation/emission wavelengths. Accordingly, we registered the spectrophotometric profiles of mCherry and the firefly luciferase, observing the same excitation/emission wavelengths reported before (Shaner et al. 2004), indicating that fusing the luciferase and mCherry together did not affect the *in vitro* performance of the fluorescent protein (data not shown). We next corroborated these data by testing the *in vitro* functionality of the cloned fluorescent protein (Fig. 1.4A). We performed several transfections with growing molar ratios of our vector (E-M-H-mCherry-Luc) along with an expression plasmid enconding the HIF-1α transcription factor (pcDNA3-HIF-1α) into the human HEK 293 cell line.

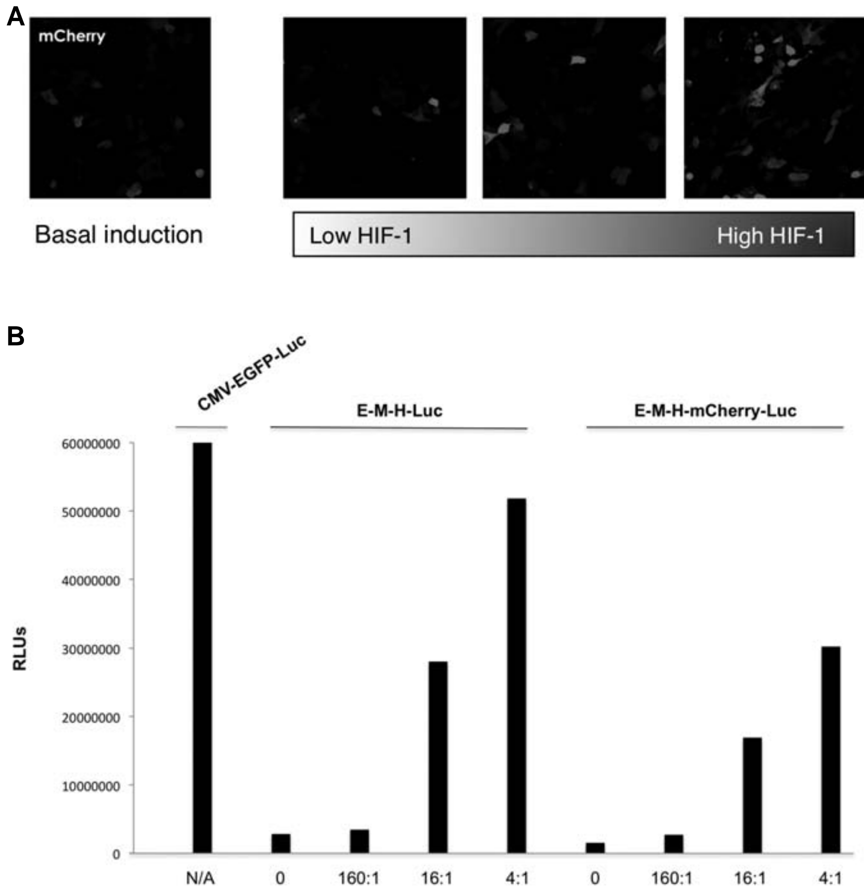

Figure 1.4. (A) *In vitro* fluorescence activity of the biosensor in HEK 293 cells. Basal activity denotes induction due to endogenous HIF-1α levels. Cells were transfected with increasing quantities of a plasmid encoding HIF-1α. **(B)** Luciferase activity of the fusion protein for E-M-H-Luciferase and E-M-H-mCherry-Luciferase.

As expected, the fluorescence signal observed was proportional to the quantity of HIF-1α transfected with the biosensor, indicating that the system is proportionally responsive to the amount of transcription present in each assay.

Next, we tested the luciferase activity and performance of the system *in vitro*. As shown in Fig. 1.4B we observed the same proportionality of the bioluminescent signal in relation with the concentration of the transcription factor HIF-1α. Intriguingly, we observed that one of the control groups

where the parental vector E-M-H-Luc was transfected, displayed a similar signal than that of the biosensor with the fusion protein. This discrepancy hinted of a probable energy transfer between FLuc and mCherry that we later confirmed, and to rule out any non-expected hindrance of the luciferase catalytic site caused by mCherry that would result in a weaker FLuc activity. As means of transfection normalization and to rule out discrepancies on transfection efficiency in the different experimental groups, all data were normalized against the β-galactosidase activity of each group.

Subsequently, we wondered if this fluorescent and bioluminescent performance could be translated into an *in vivo* environment. Therefore, we next co-transfected HEK 293 cells with our biosensor and either pcDNA3-HIF-1α, or a transfection control (pcDNA3). Thus, we obtained two groups of cells that contained either the activated biosensor (with artificially high levels of HIF-1α) or non-activated cells that would only display a basal activity due to endogenous HIF-1α. Upon confirming the optical activities of these cells both groups were injected subcutaneously as xenografts in the hindquarters of immunodeficient SCID mice.

Fluorescent signals were measured 24 hr upon injection as shown in Fig. 1.5A . Accordingly, we confirmed that the system remains active in the xenografts formed by HEK 293 cells transfected with the E-M-H-mCherry-Luc (ECL) biosensor, and that their intensity directly correlates to the quantity of the transcription factor HIF-1α present in those cells. Likewise, luciferase activity was also registered *in vivo* (Fig. 1.5B). Upon administration of D-luciferin, we observed a similar output as before with both xenografts, activated with HIF and non-activated, emitted bioluminescent light but with higher intensity in the case of cells with the activated system. Taken

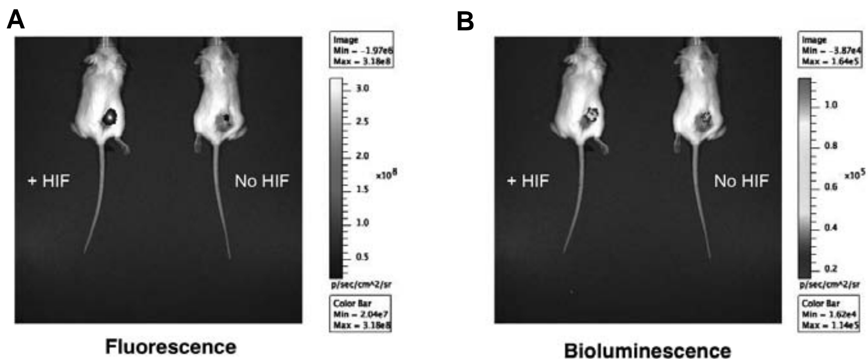

Figure 1.5. (A) *In vivo* mCherry fluorescence in a xenograft implanted subcutaneously in SCID mice. The left mouse carries the "activated biosensor" ('+ HIF'), HEK 293 cells transfected with E-M-H-mCherry-Luciferase and pcDNA3-HIF-1α while the right mouse was injected with the 'non-activated' system ('No HIF') in cells transfected with E-M-H-mCherry-Luciferase and pcDNA3 as a negative control. **(B)** *In vivo* luciferase activity in SCID mice. Left mouse carries the activated biosensor and right mouse the no-activated one.

together, these data demonstrate that our hypoxia biosensor is able to proportionally induce the transcription of the mCherry-luciferase tracer when the concentration of HIF-1α is high enough to bind the response elements located upstream to the fusion protein coding sequence. A similar response was also observed both *in vitro*, in HEK 293 cells transfected with increasing concentrations of HIF-1α, and *in vivo* xenografts of these transfected cells, as shown in Fig. 1.5A and Fig. 1.5B.

As discussed before, we observed a marked decrease when registering the bioluminescent activity of the biosensor and comparing it to that of the parental vector E-M-H-Luc (Fig. 1.4B). We hypothesized that it could be either a consequence of a resonant energy transfer (RET) between FLuc and mCherry, or that by fusing those two proteins together the catalytic site of FLuc resulted in reducing the enzyme's ability to bind to its substrate. Consequently, we first tested whether or not this transference was taking place *in vitro* by comparing the spectrophotometric profiles of whole cell lysates of cells transfected with the parental vector (E-M-H-Luc) or with our biosensor (E-M-H-mCherry-Luc). As shown in Fig. 1.6A , the luciferase alone

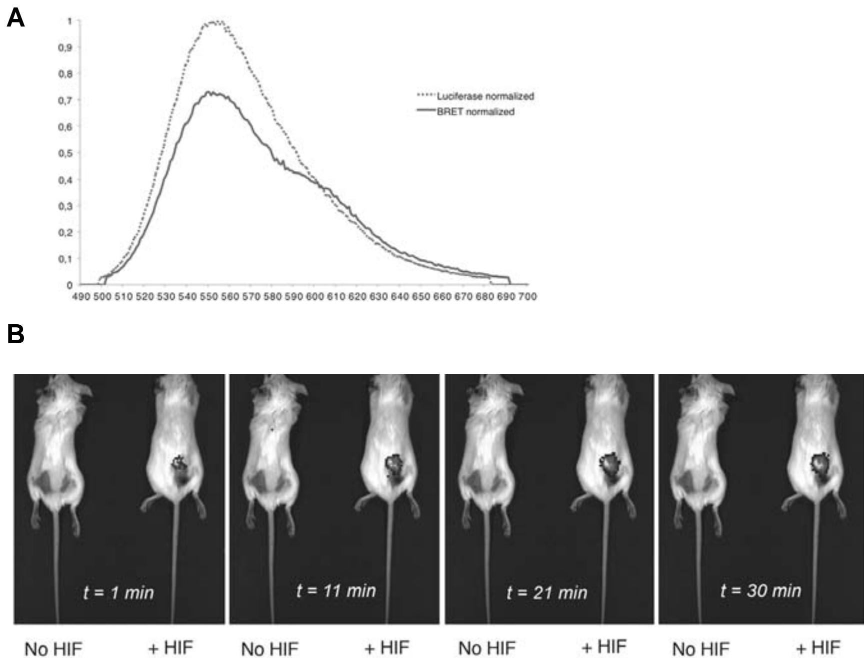

Figure 1.6. (A) *In vitro* BRET performance of the genetically encoded biosensor. Solid line represents luciferase activity of the E-M-H-Luciferase vector; dashed line represents luciferase activity of the E-M-H-mCherry-Luciferase vector. **(B)** *In vivo* BRET performance of the system at various times. Maximum BRET emission was reached 30 min upon injection of D-luciferin (150 mg/kg).

displays a maximum value at the expected wavelength of 575 nm. However, it also displays a lower second peak at a wavelength corresponding to that of mCherry emission maximum wavelength. In addition, a fall of the bioluminescent activity was also observed in this case that would be consistent with the existence of BRET, as the second emission peak suggests. This would imply that part of the bioluminescent light would be absorbed by the fluorochrome rather equalling the difference between both peaks registered by the spectrofluorometer detector.

With this in mind and once we had demonstrated that BRET could be registered *in vitro* in whole cell lysates we wanted to confirm whether or not this phenomenon was also occurring in xenografts in SCID mice. We finally investigated whether or not this BRET phenomenon could be also detected *in vivo*. We consequently registered the fluorescent emission of the activated system while at the same time blocking the excitation filter in order to avoid any excitation source other than the FLuc luciferase. Figure 1.6B shows some of the most representative points of the series including the peak emission of BRET in SCID mice at 30 min upon D-luciferin injection.

CONCLUSION

Fluorescence and bioluminescence imaging are experiencing slow but steady advances to become another tool in clinical environments. These kind of optical methods provide a functional insight that is rarely achieved using other traditional yet spatially powerful techniques such as CT or MRI. As an addition to these optical methods, BRET can be considered an emerging technique that has the best from both fluorescence and bioluminescence. Given that BRET does not depend on an external source of excitation but rather on a chemical reaction it does not present the characteristic tissue autofluorescence that is common in living organisms, and at the same time, the fluorescent light emitted by the fluorochrome is better suited for 3D tomographic reconstructions than bioluminescent light, at the same time allowing its application at deeper locations than fluorescence alone (Dinca et al. 2010). We have described several examples of biosensors used primarily in oncology that not only highlight the tumoural mass but at the same time also inform us on specific biological processes related to tumoural development and maintenance. We have also described the development and characterization of a BRET-based hypoxia biosensor that uses the firefly luciferase (FLuc) as a bioluminescence donor and a NIR fluorochrome, mCherry, as fluorescent acceptor. This genetically encoded biosensor is induced by the hypoxia transcription factor HIF-1α, which acts as key regulator of the angiogenic switch of tumoural cells in response to hypoxic conditions. We have demonstrated that this factor binds the response element located in the regulatory module of the construction

efficiently inducing the transcription of the fusion protein, displaying at the same time a proportional response to the concentration of HIF-1α within the cells carrying the biosensor. Although the development of a BRET-based biosensor (So et al. 2006) is not a novelty in the field, combining NIR fluorescence and bioluminescence results in a valuable alternative approach for future inducible biosensors that take advantage of BRET.

KEY FACTS

- The green fluorescent protein (GFP) was first cloned in 1992 from the jellyfish *Aequorea victoria*, by Douglas C. Prasher. This wild-type GFP had lower excitation and emission peaks than their engineered counterparts used currently, mainly EGFP (enhanced green fluorescent protein).
- The sea pansy (*R. reniformis*) luciferase emits light in the blue part of the spectrum at 480 nm when reacts with coelenterazine h, while the *Photynus pyralis* FLuc is a yellow luciferase emitting at 560 nm.
- One of the first applications of BRET was to investigate the dimerization of cyanobacterial circadian clock proteins in bacterial culture. The pair donor-acceptor used back then eventually became one of the most used, *Renilla reniformis* luciferase (RLuc) as donor and GFP as acceptor.
- Optical imaging is already being used as intraoperative aid for surgeons aiming to remove regional lymph nodes or metastasis, both in breast and ovary cancer respectively, improving the clinical management of these tumours and therefore their prognosis.
- Indocyanine green (ICG) is a synthetic dye traditionally used for medical diagnoses because of its ability to penetrate in deeper tissues in angiography studies. Currently, it is being tested for assessing vascular leakage associated with tumour invasion.

DEFINITIONS

- *Bioluminescence*: production of light as a result of a chemical reaction catalysed by a class of enzymes denominated luciferases, found in multiple living organisms.
- *Fluorescence*: physical phenomenon where an external source of light excites a molecule, called fluorochrome, and this emits light at a higher wavelength.
- *BRET*: Bioluminescence resonance energy transfer, a photophysical phenomenon occurring between a donor-acceptor pair where the donor is a light emitting luciferase (RLuc or FLuc) and the acceptor

a fluorochrome excited by the bioluminescent emission of the luciferase.

- *D-luciferin*: (4*S*)-2-(6-hydroxy-1,3-benzothiazol-2-yl)-4,5-dihydrothiazole-4-ca rboxylic acid. It is the substrate of the firefly luciferase that in the process becomes oxydized (oxyluciferin) and emits the characteristic yellow light.
- *Coelenterazine*: 6-(4-hydroxyphenyl)-2-[(4-hydroxyphenyl)methyl]-8-(phenylmethyl)-7H-imidazo[3,2-a]pyrazin-3-one. It is the substrate of several luciferases found in aquatic microorganisms such as *Renilla reniformis* and *Gaussia princeps*.
- *HIF-1*: the hypoxia inducible factor-1 transcription factor is a heterodimer formed by the alpha and beta subunits. It is part of a family of transcription factors characterized by their basic helix-loop-helix (bHLH) DNA binding domain.
- *VEGF*: the vascular endothelial growth factor is a transcription factor involved in the formation of new vessels primarily during embryo development (*de novo* formation) but also in angiogenesis (from an existing vessel).
- *Quantum dot*: nanocrystal structures with semiconductor properties that can display fluorescent activity with emission peaks in the red-near infrared part of the spectrum.

SUMMARY POINTS

- Fluorescence and bioluminescence imaging are experiencing slow but steady advances to become another tool in clinical environments.
- These imaging techniques provide a functional insight that is not always achieved with other spatially powerful methods such as CT or MRI.
- BRET does not depend on an external source of excitation but rather on a chemical reaction, which facilitates the construction of self-illuminating probes for *in vivo* imaging.
- Functional imaging allows the visualization of biological processes associated with characteristic features of the disease, e.g. metastatic disease.
- We have designed a genetically encoded biosensor that serves as a valuable proof of concept and test benchmark for future hypoxia sensing probes based on small molecules or nanodevices powered by BRET.

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ABBREVIATIONS

BLI	:	Bioluminescent Light Imaging
BLIT	:	Bioluminescent Light Imaging Tomography
BRET	:	Bioluminescence Resonant Energy Transfer
CT	:	(X-Ray) Computed Tomography
ECL	:	E-M-H-mCherry-Luc
E-M-H	:	EGR-MRE-HRE
EGR	:	Early Growth Response
FLI	:	Fluorescent Light Imaging
FLIT	:	Fluorescent Light Imaging Tomography
FLuc	:	Firefly (*Photynus pyralis*) luciferase
FRET	:	Fluorescence Resonant Energy Transfer
GFP	:	Green Fluorescent Protein
HRE	:	Hypoxia Response Element
HIF-1	:	Hypoxia Inducible Factor-1
ICG	:	Indocyanine Green
MRE	:	Metal Response Element
MRI	:	Magnetic Resonance Imaging
NIR-F	:	Near Infrared Fluorescence
PET	:	Positron Emission Tomography
RLuc	:	Sea pansy (*Renilla reniformis*) luciferase
SPECT	:	Single Photon Emission Computed Tomography
VEGF	:	Vascular Endothelial Growth Factor

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2

Use of a Surface Plasmon Resonance (SPR) Biosensor to Characterize Zwitterionic Coatings on SiO_2 for Cancer Biomarker Detection

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ABSTRACT

The recent push to discover new cancer therapeutics from human matrices, such as serum or plasma, has been limited by the inability to fully verify low concentration biomarkers as being clinically relevant. For biosensing platforms to successfully aid in the development pipeline, they must meet several major design requirements. First, the sensing device must possess high sensitivity with the ability to resolve small changes in analyte binding, preferable in real-time. Second, due to the abundance of irrelevant proteins in serum (or plasma), the platform must effectively reduce non-specific protein adsorption. Finally, in order to possess high sensitivity and specificity, the sensing surface must

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List of abbreviations after the text.

also enable the efficient immobilization of biorecognition elements for detecting target analytes with high affinity.

Advances in microelectromechanical system process technologies have led to numerous micro- and nano-scale biosensing platforms based on silicon substrates. Therefore, we briefly review current approaches for creating protein-resistant surface coatings using both "graft-from" and "graft-to" approaches. The recent development of zwitterionic polymer conjugates containing two carboxybetaine methacrylate (CBMA) polymers linked to two 3,4-dihydroxy-L-phenylalanine (DOPA) residues provides a convenient and simple approach for forming protein-resistant coatings on silica substrates as determined with a surface plasmon resonance biosensor. The zwitterionic nature of CBMA polymers significantly reduces protein adsorption while abundant carboxylic acid moieties provide for efficient antibody immobilization using common amino-coupling chemistries. By combining the adhesive properties of DOPA with zwitterionic CBMA, we introduce a new "graft-to" technology for cancer biomarker detection and show its ability to sensitively detect a model protein directly from undiluted human serum.

The transferability of the new "graft-to" technology enabled by zwitterionic polymer conjugates onto the sensing platform of a Si-based biosensor is demonstrated with a suspended micro-channel resonator (SMR). Successful *in situ* polymer attachment and antibody functionalization led to the detection of a cancer biomarker directly from complex media for the first time with a SMR device. The application of this new approach to other areas in the biomedical field, including nanoparticles for theranostic applications, illustrates the great potential for this novel surface coating.

INTRODUCTION

The mantra, "no biomarker, no drug" is frequently heard in a pharmaceutical industry heavily concerned with developing new cancer therapies (de Bono and Ashworth 2010). While some provide a specific target for selective cell cytotoxicity, biomarkers (e.g. proteins, metabolites, DNA, etc.) also play a vital role in early disease diagnostics, monitoring the therapeutic response, and improving patient stratification for treatment. The extreme complexity and heterogeneity of cancer both between patients and between cells within a patient has led to a continued push for discovering more biological indicators. However, the major shortcoming in the development pipeline is the inability of biosensors to fully verify these analytes as reliable targets resulting in few candidates reaching the patient's bedside (Brennan et al. 2010; Kulasingam et al. 2010).

Cancer biomarkers can be identified from several sources including human bodily fluids, tumor tissues, human cancer cell lines, and animal models. Due to the minimally invasive accessibility, abundance, and the ability of the circulatory system to reflect numerous and dynamic pathological states, plasma and serum are the most common choices for analysis. However, the complexity of these media (concentrations that span over 12 orders of magnitude with 22 proteins representing 99% of the total protein mass) has severely hindered the sensitive detection of biomarkers which are typically present at ng/mL to pg/mL quantities. Consequently, this has led to their inability to be verified as clinically relevant (Kulasingam et al. 2010).

These shortcomings have given rise to several major requirements for biosensor design. The low concentration of target analytes in complex media requires that the device possess high sensitivity thereby allowing for the quantification of small changes in response, preferably in real-time. Due to the presence of many irrelevant proteins, the platform must also effectively reduce non-specific protein adsorption (i.e. biofouling) from the plasma (or serum). Last, the biosensing surface must be able to sufficiently immobilize molecular recognition elements (e.g. antibodies) in order to provide highly specific detection (Vaisocherova et al. 2009). The ability to meet these challenges will better enable the verification of cancer biomarkers with high sensitivity and specificity.

In the following, we present a brief overview of current biosensing technology along with several protein-resistant materials frequently used in detection assays. As a result of the numerous advances in microelectromechanical system (MEMS) process technologies, many micro- and nano-scale biosensing devices are based on silicon (Hunt and Armani 2010; Libertino et al. 2009). Therefore, approaches for creating non-fouling surfaces on Si-based platforms are specifically addressed. Recently, the development of zwitterionic polymer conjugates containing the adhesive moiety, 3,4-dihydroxy-L-phenylalanine (DOPA), has enabled a new "graft-to" technology for the convenient attachment of non-fouling materials onto SiO_2 surfaces (Brault et al. 2010). Subsequent immobilization of antibodies onto the adsorbed polymer conjugates lead to the highly sensitive and specific detection of cancer biomarkers directly from undiluted human serum using a surface plasmon resonance (SPR) biosensor. The capability of attaching this material onto the SiO_2 surfaces of other micro/nano-scale platforms, thus indicative of the diagnostic potential for these zwitterionic conjugates, is then demonstrated with a suspended micro-channel resonator (SMR) (von Muhlen et al. 2010). We conclude with the future outlook of this new polymer system for additional applications in the biomedical field.

BIOSENSORS

While key components of biosensors include the microfluidics for sample delivery and the detection assay itself, the primary difference between platforms are the physical properties which are used for monitoring biomolecular interactions. For example, SPR sensors monitor changes in the optical properties of reflected light (Homola 2008). Others can measure changes in mechanical properties, such as the frequency of vibration for the SMR (Burg et al. 2007), or even monitor differences in electrical properties (e.g. conductance) as for field-effect transistors (FETs) (Zheng et al. 2005). Despite the apparent differences between biosensing devices, each technique can provide the high sensitivity necessary for cancer biomarker detection.

SPR Biosensors

SPR biosensing is an affinity-based optical technique that enables label-free and real-time detection of biomolecular interactions. Figure 2.1A shows a SPR platform which adapts the Kretschmann geometry of the attenuated total reflection configuration with wavelength modulation. The sensing chip consists of a glass slide coated with an adhesion-promoting titanium film (~2 nm) followed by a SPR active gold layer (~48 nm). Several materials, such a thin polymer films, are typically attached to the gold surface to provide the antibody immobilization and protein resistant background necessary for detection of analytes. Additionally, the use of a multichannel flow-cell allows several independent measurements to be conducted simultaneously.

The principles of SPR biosensing based on wavelength modulation have been reviewed extensively in the literature (Homola 2008). A polychromatic light beam is first directed through a prism where it contacts the gold surface of the substrate at a fixed angle of incidence. This leads to the coupling of optical energy (i.e. light at a specific wavelength) and the creation of a surface plasmon resonance which propagates along the boundary of the metal-dielectric interface (e.g. gold and water). The specific wavelength of light used to excite the surface plasmon resonance, the resonant wavelength, is highly sensitive to refractive index changes occurring in the dielectric within close proximity to the gold surface (i.e. less than 200–400 nm). The coupling and subsequent dissipation of energy associated with a specific resonant wavelength can be detected as a narrow "dip" in the spectrum of reflected light using a spectrophotometer (Fig. 2.1B). As the refractive index increases, such as for the binding of analytes onto immobilized receptors, the resonant wavelength also increases. Thus, by monitoring changes in the reflected light, highly sensitive label-free detection in real-time is enabled.

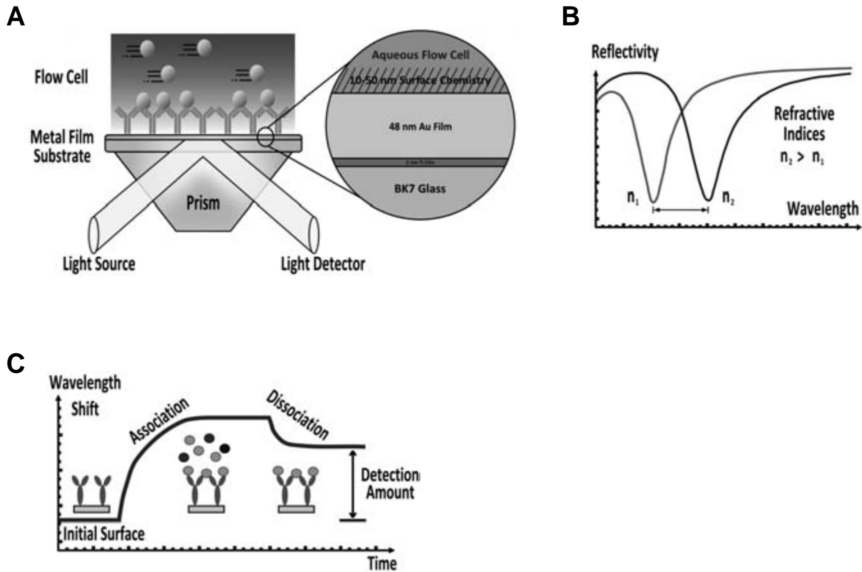

Figure 2.1. A surface plasmon resonance (SPR) biosensor based on wavelength modulation. **(A)** SPR gold substrates are typically modified with surface chemistry to enable probe molecule immobilization and a reduction in non-specific protein binding. Detection of analytes proceeds with a polychromatic light source that gets directed through a prism and strikes the SPR-active gold layer at a fixed angle of incidence; **(B)** The specific wavelength of light which couples to the gold surface plasmon resonance is detected by a spectrophotometer as a "dip" in the reflected spectrum. As the refractive index of the dielectric (i.e. solution side of the chip) increases, the resonant wavelength also increases; **(C)** Monitoring the resonant wavelength shift enables highly sensitive and label-free detection in real-time.

Figure 2.1C depicts a SPR sensor-gram, a plot of the resonant wavelength shift versus time, for the detection of antigens binding to an antibody functionalized sensor surface. After first flowing buffer to establish a stable baseline, a solution of antigens is injected into the device. The binding (association) of analytes to immobilized probe ligands increases the refractive index at the interface which increases the resonant wavelength. Upon flowing buffer, loosely bound analytes are removed (dissociated) from the surface thereby decreasing the resonant wavelength. After a sufficient amount of time the baseline flattens out. The difference between the starting and ending buffer baselines allows the specific amount of bound analyte to be quantified and converted to a surface coverage. These SPR devices have a limit of detection of ~0.1 ng/cm^2 (Homola 2006). Due to their relative ease of use and ability to sensitively monitor biomolecular interactions, these biosensors have been applied in numerous fields including medical diagnostics, food safety, national security, and environmental monitoring.

Silicon-based Micro/Nano-Biosensors

Emerging biosensing configurations have sought to create micro-scale and even nano-scale platforms with the goals of improving the detection sensitivity, decreasing the sample volume, and manufacturing portable devices. These small scale sensors also allow for reduced diffusion path lengths and overall faster kinetics (Libertino et al. 2009). To achieve these outcomes, many researchers have been focusing on Si-compatible systems due to the maturity of silicon technology in the semiconductor industry and the advances in MEMS processing techniques for this material. A variety of micro- and nano-scale biosensors have been developed based on different transducing mechanisms utilizing silicon. Several examples include the SMR, single silicon nanowire arrays, and micro-ring resonators, which are based on mechanical vibrations, electronic FETs, and photonic resonance, respectively (Burg et al. 2007; Ramachandran et al. 2008; Zheng et al. 2005).

SMR biosensors are composed of a small microfluidic channel which passes through a resonating cantilever vibrating in a vacuum. The adsorption of biomolecules onto the sensor surface decreases the resonating frequency thereby enabling label-free detection of analytes with sub-femtogram resolution. The dimensions of the micro-cantilever are 200 x 33 x 7 μm^3 (length x width x thickness) with channels containing a cross-sectional area of 3 x 8 μm^2 (Burg et al. 2007). Silicon nanowire-based FET biosensors use a single Si-nanowire as a gate between the source and drain on SiO_2 on Si substrates. Charged proteins that adsorb onto the functionalized silicon nanowire induce a change in the source-drain conductivity at a fixed gate voltage thereby enabling detection with picogram sensitivity (Zheng et al. 2005). However, both of these examples represent a common disconnect within the biosensing field; an increase in sensor sensitivity does not necessarily translate to an increase in assay sensitivity. The inability to effectively reduce non-specific protein adsorption from undiluted human plasma (or serum) onto sensing surfaces has severely limited the full exploitation of these devices. Due to the presence of a native oxide layer on silicon surfaces upon exposure to air, it is desirable to develop convenient and effective surface coatings for SiO_2 in order to provide the non-fouling background necessary for highly sensitive and specific detection from complex media.

NON-FOULING SURFACE COATINGS

Non-specific protein adsorption onto surfaces (i.e. biofouling, Fig. 2.2A) primarily occurs via hydrophobic or electrostatic interactions (Ostuni et al. 2001). For biosensing, this can lead to an overwhelming background noise

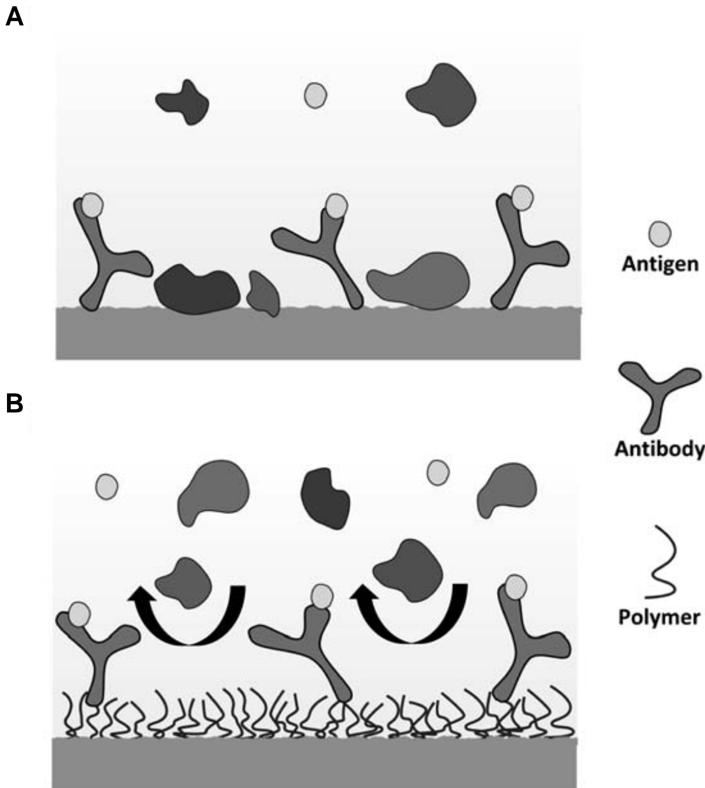

Figure 2.2. Biofouling on sensing surfaces. **(A)** Non-specific protein adsorption (i.e. biofouling) onto a sensing surface occurs primarily via hydrophobic or electrostatic interactions. For biosensing, this generates a large background noise and results in reduced detection limits and false-positives. **(B)** Protein resistant surface coatings reduce non-specific adsorption due to the presence of a hydration layer (i.e. a strongly structured layer of water) and thereby improve the signal-to-noise ratio necessary for highly sensitive and specific detection.

which decreases the detection sensitivity of low concentration biomarkers and increases false-positives. Protein-resistant surface coatings (Fig. 2.2B) can significantly reduce non-specific adsorption due to the presence of a hydration layer (i.e. a strongly structured water interface) thereby improving the signal-to-noise ratio and increasing the detection sensitivity and specificity (Chen et al. 2005). Several methods for reducing protein adsorption on biosensing platforms have been implemented.

Material Selection

Blocking agents (e.g. BSA, fish gelatin, etc.) and surface coatings are commonly employed in biosensors in order to reduce or eliminate fouling

from complex media. The use of blocking agents suffer from several limitations including cross-reactivity with assay components and only a minor reduction in non-specific binding. This has given rise to surface coatings which have been found to be both more effective and robust. Ethylene glycol (EG) and its derivatives, such as poly(ethylene glycol) (PEG) or oligo-ethylene glycol (OEG) are the most commonly used materials for preventing protein adsorption. An example of an EG-based material is shown in Fig. 2.3A. Experimental evidence indicates that a combination of tightly bound hydrogen bonding-induced hydration layers, which generate a large repulsive force, in addition to steric effects of longer PEG chains are responsible for the non-fouling properties of this material (Jiang and Cao 2010). However, while these properties enable EG to resist fouling from diluted protein solutions (e.g. plasma diluted with PBS), it is only partially effective against 100% human serum (or plasma) (Ladd et al. 2008).

Despite its benefits, the use of EG for detection of cancer biomarkers underscores a vital criterion for biosensing; in order to obtain highly sensitive detection, the immobilization of molecular recognition elements (e.g. antibodies) must be both efficient and occur without negatively affecting the protein resistance of the surface coating. Relatively complex

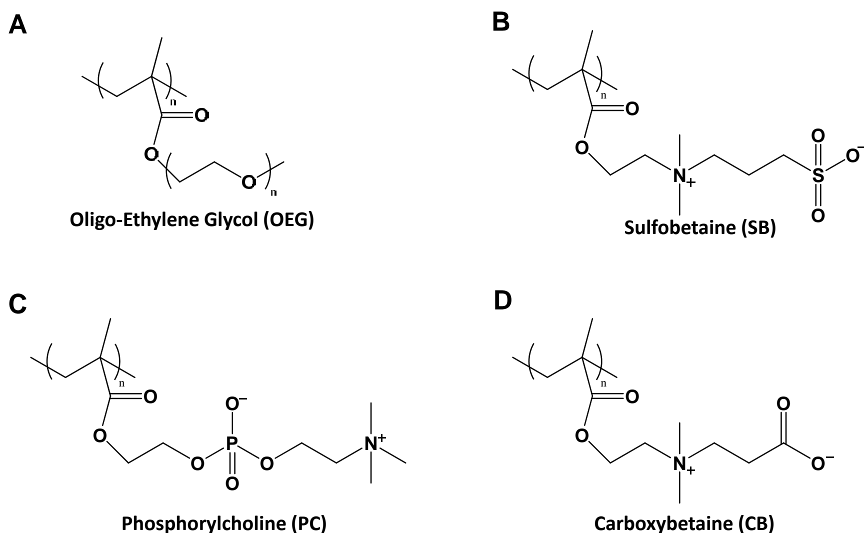

Figure 2.3. Protein resistant materials. Ethylene glycol based coatings resist protein adsorption by forming a tightly bound hydrogen-bonding induced hydration layer that provides a repulsive force against non-specific binding. **(A)** Zwitterionic materials such as sulfobetaine **(B)**, phosphorylcholine **(C)**, and carboxybetaine **(D)** offer improved protein resistance by achieving much stronger hydration via electrostatic interactions. (Adapted from Jiang and Cao. Copyright 2010 Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.)

chemical reaction steps are necessary to functionalized EG-based materials. Furthermore, the use of blocking agents to pacify unreacted activated groups is also necessary. Both of these requirements typically result in worsening the non-fouling properties and thereby limit the overall effectiveness of this material for biosensing applications (Hucknall et al. 2009).

Recently, zwitterionic polymers have been shown to offer improved protein resistance from complex media compared to that of EG. These materials, such as sulfobetaine (SB), phosphorylcholine (PC), and carboxybetaine (CB) as shown in Fig. 2.3B-D, achieve much stronger hydration via electrostatic interactions, which provides the primary physical mechanism for improved non-fouling properties (Chen et al. 2005). It has been demonstrated that highly dense zwitterionic polymer films with controlled lengths exhibited undetectable adsorption to both single protein solutions and undiluted human serum and plasma using a SPR biosensor (Yang et al. 2009). Furthermore, the presence of a carboxylate group in each monomer of CB polymers enables the convenient attachment of antibodies via common N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide /N-hydroxysuccinimide (EDC/NHS) amino-coupling chemistry. The hydrolysis of unreacted NHS-esters simply converts the activated groups back into the original non-fouling zwitterionic background without the use of blocking agents. This has been shown to result in highly sensitive cancer biomarker detection (~10 ng/mL) directly from undiluted human plasma using thin films of CB polymers formed via surface initiated-atom transfer radical polymerization (SI-ATRP) (Vaisocherova et al. 2008).

Protein Resistant Coatings for Oxide Surfaces

The nearly continuous design and manufacture of novel Si-based sensor platforms with unprecedented sensitivity have made non-fouling surface coatings for SiO_2 surfaces highly desirable. In order to coat silica surfaces with thin films of protein-resistant zwitterionic polymers, two methods can be adopted, "graft-from" and "graft-to", as shown in Fig. 2.4 (Currie et al. 2003). For the "graft-from" approach (Fig. 2.4A), the original silica substrate is first modified with a monolayer of silane initiators to introduce the reactive group (e.g. bromine) necessary for subsequent polymerization (Zhang et al. 2006). After adding the appropriate monomer and catalysts, the polymerization reaction proceeds allowing for the formation of dense films over a wide range of controlled thicknesses (Yang et al. 2009). Zwitterionic polymers "grafted-from" glass substrates via SI-ATRP have been previously shown to be highly resistant to protein adsorption (Zhang et al. 2006).

The "graft-to" approach (Fig. 2.4B) enables the convenient attachment of protein resistant surface coatings by exposing the substrate to pre-synthesized polymer conjugates composed of a non-fouling polymer