[Frontiers in Bioscience E4, 101-110, January 1, 2012]

Bioconjugated nanomaterials on devices for infectious disease diagnostics

Concepcion Ponce[1], Philip Ian Padilla[2, 3]

[1]Department of Chemistry, College of Arts and Sciences, University of the Philippines, Visayas, Miag-ao, Iloilo, Philippines, [2]Division of Biological Sciences, College of Arts and Sciences, and the National Institute of Molecular Biology and Biotechnology, University of the Philippines, Visayas, Miag-ao, Iloilo, Philippines; [3]Dept of Molecular Epidemiology, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan

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1. ABSTRACT

The successful use of the nanoscale-dependent properties of nanomaterials on infectious diseases diagnostics devices lies on a well-engineered surface of both the probes and the transducer. Engineering probe and transducer surfaces involve functionalization of nanomaterials, conjugation to biorecognition elements and nanopatterning. This review presents the most common and most promising functional groups, bioconjugation schemes and patterning strategies of nanomaterials on sensing devices and their specific application to infectious pathogen detection.

2. INTRODUCTION

Nanomaterials have structural features and properties in between those of single atoms/molecules and continuous bulk materials and have at least one dimension in the nanometer range (1 nm = 1 x 10^{-9} m). They include clusters, nanoparticles, nanocrystals, quantum dots, nanowires, nanotubes, as well as the collection or organization of these individual structures into two- and three-dimensional assemblies (1-9). The optical, electronic, magnetic, catalytic and other properties of nanomaterials are distinct from those of atoms/molecules or bulk materials and have been shown to be due to their

nanoscale dimensions. In order to exploit the special properties that arise due to the nanoscale dimensions of materials, researchers have to learn how to control and manipulate the size and shape of nanomaterials and structure them into periodically ordered assemblies to create new products, devices and technologies or improve existing ones (10-17). The art of controlling/manipulating the properties and utilizing these nanomaterials for the purpose of building microscopic machinery is termed as nanotechnology. The control and manipulation process can be done using the "top down" or "bottom up" approach. In the "top down" approach, large chunks of materials are broken down into nanostructures by lithography or any other outside force that impose order on nanomaterials (18).The "bottom up" approach, on the other hand, tries to follow nature's lead in making extraordinary materials and molecular machines by building nanostructures from atoms or molecules or any stable building blocks through the understanding and exploitation of order-inducing factors that are inherent in the system (19). The last two decades has seen an increase in the understanding of the factors that govern growth and ordering of nanomaterials which has led to a number of novel structures and functionalities. For example, the mechanism of formation/fabrication of novel nanostructures from self-assembly of peptides, proteins and lipids has been elucidated as described in a review article by Zhang (19). These nanostructures include nanofibers, bionanotubes, amphiphilic protein scaffolds and nanowires that have potential applications in the electronic industry, biomedical field, computer information technology, etc.

The application of nanomaterials and their assemblies in the biomedical field especially in diagnostics is still in its infancy but is gaining ground fast. As proof of the increasing importance of nanotechnology in the field of medical diagnostics, a number of review articles have already been written about the topic. Jain focused on the type of nanomaterials used in various biosensor technologies with emphasis given to quantum dot (QD) technology for diagnostics (20). Driskell and Tripp, on the other hand, focused on nano-transducers or the application of nanomaterials to different platforms for signal detection (optical, electrical and mechanical) (21). Kaittanis discussed the different strategies for different disease diagnostics and how nanotechnology can revolutionize these strategies (22). Lee reviewed recent advances in nano/microfluidic technologies for clinical point-of-care applications at resource-limited settings in developing countries (23). Hauck described the use of nanomaterials as labels or barcodes in conjunction with microfuidic systems for automated sample preparation and sample assays for infectious diseases diagnostics (24).

It appears that there is a plethora of scientific articles dedicated to nanotechnology in medical diagnostics (20-24). However, most of the articles mentioned focused on either the types of nanomaterials used as probes and transducer components of the biosensor or on the impact of nanomaterials on current medical diagnostic strategies (20-23). We recognize that the successful use of the nanoscale-dependent properties of nanomaterials for medical diagnostic devices lie on a well-engineered surface of both the probes and the transducer. For example, in amperometric biosensors, a fast and highly sensitive detection is dependent on a fast electron transfer from the biological recognition element to the electrode. This requirement, thus, spurred a number of studies devoted to the design of different electrode surface architectures that aimed to optimize the response of amperometric biosensors (25). In surface enhanced Raman scattering (SERS), inherent high sensitivity and the ability to provide unique spectroscopic fingerprints of the target analyte make it a potent biosensing technique, but then again, appropriate substrate engineering must be addressed first before it can be successfully employed for routine biosensing applications (26).Thus far, we have cited two examples where engineering the transducer surface (*i.e.* by nanopatterning) to improve biosensing performance is important. In most biosensing devices, the biological recognition element or the bioreceptor is the subject of molecular engineering studies because they are the ones that provide selectivity (they are chosen based on their response to only one analyte thus eliminating interferences from other substances) to the biosensor. However, most biological recognition elements such as DNA, peptides, proteins and aptamers do not produce a strong signal for transduction and need to be attached without losing its activity to the transducer surface. This requirement to attach a bioreceptor to a nanomaterial probe or transducer surface is another active area of molecular engineering research. In this paper, we will revisit the different methods of engineering the surface of nanomaterials to allow attachment/conjugation of a bioreceptor. We will also look at techniques of nanopatterning the biosensor surface to optimize sensing performance and put emphasis on the techniques that have already been applied or has great potential in infectious diseases sensing devices. A brief introduction on the general components of a biosensor and where nanomaterials play a significant role will be discussed before the engineering techniques will be presented.

3. BIOSENSORS

A biosensor comprises a biorecognition element, which interacts with the target molecules, and a transducer, which detects the interaction and converts the binding event to a measurable analytical signal. Biosensors are classified according to the bioreceptor used or biological process involved such as biocatalytic (enzyme-based), immunologic (antibody-antigen based) or DNA (nucleic acid-based) sensors (25,27-33). They can also be classified according to the type of signal transduction such as optical, electrical, thermal or mechanical (21,34-37). Biosensors are often developed for detecting target molecules in clinical samples but a number of biosensors also exist for ensuring food quality and safety; for monitoring and control of environmental contaminants and for addressing agricultural problems (35-39).

3.1. Functionalization and bioconjugation of nanomaterial probes

Nanomaterials such as nanoparticles (gold, silver and silica), magnetic beads, quantum dots, carbon

nanotubes and semiconductor and polymer nanowires are often attached to bioreceptors for biosensing applications (21,30). Their surfaces are often functionalized to allow them to interact selectively/specifically with biomolecules and to facilitate self-assembly and nanopatterning on biosensor surfaces.

Surface functionalization of nanomaterials is carried out with the use of stabilizers that are added during the synthesis process (*in situ* functionalization). These molecules are attached to the surface of the nanomaterials by physisorption or by ligand-like interactions as in the case of sulfur containing molecules as discussed in various review articles (39-41). Below are examples of compounds that give surface functionalities to different nanomaterials.

Gold nanoparticles (AuNPs) prepared in the presence of aqueous citrate gives citrate functionalized gold nanoparticles (39,42). The citrate is attached to the surface of the gold only loosely which make it an important precursor in preparing other functional gold nanoparticles through a ligand exchange process. The Brust-Schiffrin method functionalized the gold nanoparticle surface with thiol ligands as discussed in a review by Daniel and Astruc (39). Other examples of ligands and physisorbed compounds that are used in the stabilization and functionalization of gold include phosphines, phosphine oxides, polyethylene glycol, carboxylates, amines and perfluorophenylazides (9, 39,43-47).

High quality quantum dots are often prepared in high temperature solvents – often a mixture of trioctyl phosphine and trioctyl phosphine oxide (TOP/TOPO), then a layer of wide-bandgap semiconductor materials can be coated on the surface of the quantum dot core shell (40, 48-50). The QDs are then made water-soluble by ligand exchange or by adsorption of heterofunctional organic coating on the QD surface (40,50-53). A comprehensive review by Medintz et al. summarizes the group of compounds that give functionality to the QD surface (40). These include monothiols, bidentate thiols, silanes/silanols, amine box dendrimers, oligomeric phosphines, phosphatidyl compounds, amphiphilic saccharides, proteins and peptides etc (40-41,54-63).

Silica nanoparticles are often prepared by the hydrolysis and co-condensation of tetraethylorthosilicate (TEOS) and an amine-containing silane agent such as 3-aminopropyltriethoxysilane (3-APTS). The 3-APTS can be chemically bound to a wide range of dye molecules, magnetic NPs and luminescent quantum dots (64). Other functional groups mentioned in the review by Wang *et al.* that can be coated to the initial silica NP include amines, thiols, carboxylates, methacrylates (64). Yavkovleva *et al.* as cited by Ng *et al.*, however, found out that in the case of silicon substrates, longer and flexible linkers like polyethyleneimine (PEI) and dextran (DEX) are more favorable than the shorter 3-APTS as these addressed problems on antibody accessibility and steric hindrances (65).

Magnetic nanoparticles are chemically prepared from the high temperature decomposition of organometallic precursors in the presence of surfactants (4,66). The surfactants are carefully chosen such that they adhere strongly enough to the nanoparticles surface to prevent agglomeration of the NPs but that the adhesion should also be weak enough to allow on and off exchange of the surfactant on the surface of the growing crystal. Such surfactants include alkyl phosphine oxides, polymers (such as fatty acids and dextrans), silica, amines and some nitrogen containing aromatics (4,12,22,67-68).

Carbon nanomaterials are functionalized by simply oxidizing the surface of the material or treating it with plasma (70-71). Depending on the content of the plasma, the resulting carbon nanomaterials may have surfaces terminated with –O, –H or –F groups (71). Another functionalization scheme that was designed to preserve the interesting structural, mechanical, electrical, and electromechanical properties of CNTs and for subsequent bioconjugation to proteins (39). They used the bifunctional molecule, 1-pyrenebutanoic acid, succinimidyl ester. The pyrenyl group interacts strongly with the sidewall of CNTs by π-stacking while the succinimidyl ester provides the necessary chemistry for bioconjugation (39).

The surface functionalized nanomaterials are then conjugated to biomolecules that are used as the recognition element in biosensors (64). Conjugation can be done by ligand-like attachment usually by chemisorption to the nanomaterials core as in the case of sulfur containing groups, electrostatic adsorption (including those involving engineered proteins), hydrophobic interactions, conjugation by covalent binding of groups on both particle and the surface modifier, or through a receptor-ligand interaction as in the case of avidin-biotin system (40-41,71-77).

Among the nanomaterial bioconjugation schemes mentioned, covalent binding is preferred to avoid desorption of the biomolecule from the surface of the nanomaterials (41). Bioconjugation of nanomaterials often use the carbodiimide derivative 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for linking –COOH groups of the nanomaterials to the $–NH_2$ of the bioreceptors (33,40,64,72). The linker 4-(N-maleimidomethyl)-cyclohexanecarboxylic acid N-hydroxysuccinimide ester (SMCC) is used for linking sulfyhydryl (-SH) groups of the nanomaterials to the $–NH_2$ groups of the bioreceptor (41). One notable application of the carbodiimide chemistry in infectious diseases diagnostics is the conjugation of the pathogen biomarkers hepatitis B surface antigen (HBsAg), HCV nonstructural protein 4 (NSP4), and HIV glycoprotein 41 (gp41) to the surface of quantum dot barcodes (QdotBs) (78). Their use of the QdotBs along with advances in microfluidic and photon detection technologies, signal processing and proteomic biomarkers of infection, have shown potential to be developed as a hand held device for point of care diagnostics. Agrawal showed how carbodiimide chemistry was also used to conjugate oligonucleotides to green and red nanoparticle probes for the determination of intact respiratory syncytial virus (RSV) and that the use of bioconjugation of two-color

nanoparticles to simultaneously recognize two binding sites in a single targ*et al*lows for detection of intact viruses without the need for target amplification or the need to separate probe from target molecules (79).

Carbodiimide chemistry using 1-ethyl-3–3(3-dimethylaminopropyl) carbodiimide hydrochloride (DAPC) as crosslinker has been used to functionalize a tris(2,2′-bipyridyl) dichlororuthenium(II) hexahydrate (RuBpy)-doped silica nanoparticles for the subsequent immobilization of antibody for *E. coli* (80). The antibody bioconjugated NPs allowed for rapid and accurate detection of a single bacterium without the need for amplification or enrichment (80).

Another bioconjugation scheme used the homobifunctional coupler, 1,4-Phenylenediisothiocyanate (PDITC), to bind the 38-kda tuberculosis antigen to both the amino group of the aminopropyltriethoxysilane (APTS)-modified sensor surface and the amino groups of the antigen as well (81). The coupling chemistry allowed for specific binding but suppressed nonspecific binding of blood serum and whole blood compounds in a serodiagnostic measurement of TB infection using three optical sensing platforms (81).

The EDC or SMCC chemistries are favorite bioconjugation schemes for nanomaterials but they are not considered a universal scheme. In fact, the group of Mattousi argued against EDC conjugation of quantum dots (QDs) for the following reasons: the formation of aggregates due to instability of QDs and the various crosslinking reactions possible and the formation of large and uncontrolled number of conjugates on a single nanocrystals (40). Nevertheless, they have conceded that the EDC chemistry is widely used for the preparation of commercial QD-avidin/streptavidin conjugates with high quantum yield which in turn is used to attach different functionalities on QDs to allow for a more specific interaction with different target analytes.

Other schemes that have been used in biosensors for infectious diagnostics devices include the electrostatic interactions (40-41). For example, silver nanoparticle in polystyrene sulfonate/polyacrylic acid capsules were coated with avidin via electrostatic interactions and used as label for electrochemical detection of *Escherichia coli* DNA hybridization through a biotin-avidin interaction (82). Their use of capsules of silver nanoparticles was reported to: 1) lower the detection limit as compared to other Ag nanoparticles-based assays; 2) lower the detection limit in comparison to many hybridization assays using other nanomaterials and; 3) approach the sensitivity of chemiluminescence , which is reported to be one of the most sensitive DNA detection methods using silver nanoparticles (82).

The group of Meyer has exploited the specific biotin-avidin interaction when they used commercially prepared magnetic beads coated with streptavidin for the detection of *Yersinia pestis* and *F. Tularensis* (83-84). The detection principle used anti-Ft or anti-YPFI antibodies for capturing the Ft or YPFI antigen. Biotinylated antibodies were coupled to the streptavidin in magnetic beads. Binding resulted to a change in the induced magnetic field. These streptavidin modified magnetic beads showed promise in the development of easy, fast and highly sensitive biosensor for the detection and quantification of the infectious diseases mentioned.

Although methods for bioconjugation of nanomaterials have been in existence for some time, it is apparent that their application to infectious diseases diagnostics is limited. At present, bioconjugation of nanomaterials are done to make them as probes for imaging, as delivery vehicles for cancer drugs and as therapeutics for the removal of tumor cells (85-87). But as shown in the examples above, a number of studies on bioconjugated nanomaterials for infectious diseases diagnostics have started to emerge. These studies have shown that the use of nanomaterials as conjugates to biorecognition elements have great potential in the development of biosensing devices that are: easy to use; fast; highly sensitive (lower detection limit compared to conventional assays) and; capable of detecting analytes even in complex media like blood. Moreover, due to the small dimensions of the nanomaterials, they can be incorporated into portable hand held devices for point-of-care (POC) diagnostics.

3.2. Top down and bottom up nanomanipulation of transducer surfaces

Bioconjugation of nanomaterials for diagnostics applications have led to more selective and even more specific sensing of target analytes. However, for the development of cost effective sensing devices that allow for multiplex sensing and at the same time, provide fast and high resolution analysis (more sensitive), the transducer surface are often nano-manipulated. Nano-manipulation techniques include Electron Beam Lithography (EBL), oblique angle deposition (OAD), nano imprint lithography (NIL), nanosphere lithography (NSL), dip-pen lithography, micro contact printing (μCP) and various self assembly approaches (88-89).This section will only present sensor fabrication/modification of the most promising sensors in the field of point of care medical diagnostics – amperometric and field effect transistor (FET)-based potentiometric sensors and localized surface plasmon resonance (LSPR) and surface enhanced raman spectroscopy (SERS).

In applications where uniformity and reproducibility of surface property is very important, the expensive top down approaches like EBL is still the method of choice (90). This is due to the fact that bottom up approaches such as NIL and NSL suffer from reproducibility and reliability issues (90). Nevertheless, bottom up techniques are gaining importance owing to the low cost, simplicity and speed of the methods (90-94).

Most biosensors reported in the literature use electrochemical transduction. This is due to the low cost, high sensitivity, ease of miniaturization, low power requirements and simple fabrication of electrochemical transducers (27). Electrode materials are often made up of

carbon, gold, platinum or conducting polymers. Nanostructured electrode materials are gaining importance in electrochemical transduction since their greater surface area allow incorporation of more biorecognition elements thereby enabling lower detection limits and faster response times. Nanomaterials have unique optical, electrical, magnetic and catalytic properties that facilitates label free detection of target analytes.

Carbon nanotubes (CNTs) have been increasingly used as substrates in electrochemical sensors due to their size and interesting physical and chemical properties. CNTs as substrates for amperometric sensors can be grown via microwave plasma vapor deposition using dendrimer-templated Fe nanoparticles as catalyst (95). CNT arrays have been grown via chemical vapor deposition on platinum (71). Multi walled carbon nanotubes (MWNTs) have been deposited on glassy carbon electrode using the layer by layer (LBL) technique with polyaniline (PANI) as the oppositely charged polyelectrolyte (96).CNT in a vertically-aligned "forest" configuration can be grown by self assembly of shortened CNTs and functionalized after the assembly (70). Despite the number of ways of assembling CNTs on electrode surfaces and the well-documented electron-transfer reactions on their surfaces, the application of CNTs in amperometric sensors remains mostly limited to small molecules. Wang *et al* have shown CNT modified transducers have can detect proteins and DNA down to 1.3 and 160 zmol, respectively in 25-50 μL samples indicating great promise for PCR-free DNA analysis (97). Yet, despite this promise, little work has been done for the application of amperometric biosensors on infectious diseases diagnostics. This has been attributed to lack of works to establish that amperometric biosensors do not suffer from biofouling (34).

Potentiometric transducers are not as sensitive as amperometric transducers but architectures for immunosensing of pathogens involving the use of nanowires as field effect transistors (FETs) have been considered as viable candidates for ultrasensitive biosensing applications (31). Reliable ultrasensitive biosensors using FETs are fabricated by the expensive top down approaches such as electron beam lithography and molecular beam epitaxy and then functionalized with biorecognition elements using bottom up approaches as discussed in an article by He *et al*(98). They have also pointed out that bottom up approaches of fabricating FETs offer flexibility of surface functionalization but suffers from low reproducibility and reliability. Among the mentioned bottom up assembly of nanowires FETS are electric field and magnetic field controlled deposition, Langmuir-Blodgett method, alignment and selective growth on the device, programmed dip coating, growth substrate contact printing, blown bubble film and electroplating in nanochannels (98).

Silicon Nanowire FETs have been employed for the detection of influenza A and adenovirus in a study by Patolsky *et al.*(99). Their studies showed that single viruses can be detected directly with high selectivity and their methods allowed parallel detection of different viruses. Using virtually unpurified samples, the method exceeded the capabilities of existing methods such as PCR.

In the case of optical sensors (especially SPR-based and SERS-based), research on improving sensitivity are focused on sensor design. Conventional SPR-based or SERS-based optical sensors used planar transducer surfaces made from bulk materials or colloidal materials. Surfaces roughened with nanosized materials were found to increase the sensitivity of signal transduction. In this section, examples of nanomanipulations of the optical sensor surface to improve the sensitivity of diagnostic sensing devices are presented.

3.2.1. Nanoimprint lithography

Nanoimprint lithography (NIL) is a technique that uses a hard mould that contains nanoscale features defined on its surface. These features are embossed into a polymer material cast on the wafer substrate under controlled temperature and pressure conditions. The technique has been used in the construction of two-dimensional photonic crystals for highly sensitive, non-invasive detection of influenza virus in human saliva (100).

3.2.2. Nanosphere lithography

Nanosphere lithography involves assembly of 2D hexagonally close packed nanosphere array then thermally depositing metals on the sphere mask. The nanospheres are removed in the LSPR applications whereas they are kept intact in the SERS application. The technique has been shown to provide a sensing platform that can detect anthrax spores well below the infectious dose and with a faster detection time (101).

3.2.3. Dip pen lithography

Dip pen lithographic patterning of bioconjugated gold nanoparticles enabled detection of HIV-1 p24 antigen with a lower detection limit than conventional ELISA-based immunoassays. This patterning method can reach a level of sensitivity comparable to PCR-based assay but without the need for target amplification (23).

3.2.4. Oblique angle deposition and related methods

OAD is based on a physical vapor deposition with the substrate tilted such that the vapor arrives close to the grazing angle and causes nanorods to grow on the substrate in the direction of the deposition. The technique has been employed in creating silver nanorod arrays as SERS substrates for the detection and differentiation of three different RNA viruses – adenovirus, rhinovirus and HIV (26). Abell *et al.* showed that OAD can be applied to fabricate a microwell-arrayed SERS chip on glass (102). The resulting device provided a uniform Raman signal enhancement from well to well with a detection limit of 10^{-8} M for the Raman active molecule - 1,2-di(4-pyridyl)ethylene (BPE) solution. The utility of the device for biosensing has been demonstrated using avian influenza virus (102).

A similar bottom up method called oblique angle polymerization (OAP) for the preparation of SERS substrates was performed by the group of Malvadkar (103). It differs from the OAD in that the nanostructured

poly(chloro-*p*-xylelene) was used as template for the electroless deposition of Ag or Au. The resulting SERS substrate was reported to create reproducible SERS signal and was used to detect respiratory syncytial virus (RSV). Work is still underway to improve the performance of this sensing platform (103).

4. SUMMARY POINTS

1. Functional groups anchored to the surface of nanomaterials during synthesis (in situ functionalization) provide reactive sites for subsequent bioconjugation reactions. These functional groups often contain –SH, -COOH, -NH2 etc. that allows attachment of biomolecules through standard crosslinking chemistry such as carbodiimide, succinimide, maleidiimide and bifunctional crosslinkers; through direct attachment (hydrophobic or electrostatic interactions) and; through biodin-avidin system. All these methods are not universal and have their inherent disadvantages, thus, researches on improving functionalization and bioconjugation schemes are still very active.

2. Nanomaterials incorporated in biosensors allow for development of sensing devices that are easier to use, faster and more sensitive as compared to conventional diagnostics methods. Due to their small dimensions, they can be assembled into barcodes and high density arrays to detect multiple analytes even in miniature hand held sensing devices.

3. The assembly and patterning of nanomaterials on transducer surfaces are accomplished by top down and bottom up approaches or a combination of both. For applications requiring uniformity and reproducibility of surface properties, electron beam lithography is the method of choice but the cheaper, versatile and easier to perform bottom up approaches are becoming important.

5. ACKNOWLEDGEMENTS

The authors would like to thank Mr. Geoffrey Sanchez and Dr. M. Cynthia Goh of the Department of Chemistry, University of Toronto, Ontario, Canada for their helpful suggestions and critical reading of the manuscript and the Department of Chemistry, University of the Philippines Visayas for providing computer and internet facilities in the preparation of this review article.

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Key Words: Bioconjugation, Functionalization, Nanomaterials, Infectious Pathogen Detection, Review

Send correspondence to: Concepcion Ponce, Department of Chemistry, College of Arts and Sciences, University of the Philippines, Visayas, Miag-ao, Iloilo, Philippines, Tel: 639217773425, Fax: 635137020, E-mail: concepcion_ponce@yahoo.com

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