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Polymer – Nanoparticle Assemblies for Array Based Sensing

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Abstract

Sensing clinically relevant biomolecules is crucial for the detection and prevention of disease. Currently used detection methods tend to be expensive, time intensive, and specific for only one particular biomolecule of interest. Nanoparticle-based arrays using conjugated polymers have emerged as an analytical and potential clinical tool, allowing detection of a wide range of biomolecules using selective, not specific, sensor components. In this report, we highlight recent progress in nanoparticle - polymer sensor arrays in both the fundamental understanding of how the sensor arrays function as well as the detection of clinically relevant bacteria and cells.

Keywords

Biosensing; conjugated polymers; displacement assay; nanoparticles

Introduction

Detection of biological entities including proteins, bacteria and mammalian cells in complex fluids is important for diagnosing a wide range of diseases and disease states. Currently, there are a dozens of detection and quantification methods available including enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR), and proteomics some are used in clinical settings.[1] For example, microorganism concentrations are

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Conflict of Interest

None

typically determined by plating and culturing methods or PCR, while viral infections such as human immunodeficiency virus (HIV) can be determined through ELISA type testing as well as by PCR.[2] Newer methods involving electrochemistry[3] and mass spectroscopy[4] also can effectively determine microorganism growth.

While sensors and indicators exist for specific biomolecules, selective sensor arrays are also useful as they are comprised of a small library of reporters whose responses indicate the presence of analytes. For example, Ros-Lis and colleagues used a disposable array of chromogenic indicators with different chemical recognition properties to study the progression of aging meat.[5] To lower the overall cost of these sensors, developing reactive but somewhat unspecific sensor elements could be paired together in a displacement assay where the sensor is disrupted by the presence of the analyte of interest. Indicator displacement assays (IDAs) consist of a complex where one or more recognition elements are paired with either a colorimetric or fluorometric indicator for detection.[6] When the sensor complex is subjected to an analyte, some of the indicator is removed from the recognition element to generate a detectable signal. Sensors using IDAs are attractive as one can mix receptor elements with different indicator species, producing easily producible sensor libraries. Furthermore, the elements of the sensor can be modulated to take advantage of binding affinities and selectivities of the IDAs.

Recently, nanoparticles (NPs), especially gold nanoparticles (AuNPs),[7] have been incorporated into IDAs as recognition elements as their facile synthesis, biocompatibility, tunable size and shape have led them to be widely adoptable into biological assays. These particles can be created with sizes ranging from 2 to 50 nm and can be functionalized with monolayers of organic ligands that can contribute solubility as well as recognition elements for biomolecules (Figure 1).[8]. The monolayer of these NPs can be easily altered to provide a range of surface properties in a highly divergent fashion, enabling diverse receptors to be rapidly and efficiently produced. These NPs provide versatile scaffolds for targeting, with sizes commensurate with biomolecules, a challenging prospect with small molecule-based systems. Combinations of these gold particles with different sizes and ligands gives rise to a large library of sensing elements for IDAs. In addition, AuNPs are excellent fluorescence quenchers,[9] functioning through either a FRET (fluorescence resonance energy transfer) [10] or an electron transfer type mechanism.[11] Other metal nanoparticles can be used in these systems if the undisturbed polymer/NP complex is removed from the solution to allow only the displaced polymer to remain for analysis.[26] Overall, NP/polymer complexes provide a lower-cost complement to laboratory techniques such as ELISA and antibody assays but also might be considered a modified version of gel electrophoresis. In this review, we highlight the use of NPs with a focus on AuNPs with multivalent fluorescent polymers to create self-assembled fluorescent turn on IDA sensors for the detection of relevant biological species such as cells, bacteria, and carbohydrates.

Understanding the Polymer/AuNP Complex

To create viable biosensors, it is critical to understand the fundamental interactions between the fluorescent polymer and nanoparticles in the system. To probe the interface between these components using AuNPs, Bunz and colleagues obtained the binding constants

between an anionic poly(*para*phenyleneethylene) (PPE) conjugated polymer and a wide range of 2 nm sized functionalized AuNPs (NP1-15) in aqueous solution at various concentrations of sodium chloride.[12] This chosen group of AuNPs include various length alkyl head groups (NP1-5), cycloalkyl head groups (NP6-8 and 13), aromatic head groups (NP9-12) and polar head groups (13–15). The binding constants were derived through the fluorescence quenching of the polymer by the nanoparticles. Initial results showed that aromatic monolayer functionalized AuNPs were found to quench the fluorescence more effectively, and therefore have a higher binding affinity, than aliphatic functionalized particles. However, the 4-*t*-butyl cyclohexyl functionalized particle (NP7) was shown to have the highest binding constant amongst all particles studied, suggesting that hydrophobicity on the head group could also be of importance. Furthermore, the experiments also showed that differing concentrations of saline altered the binding constant, which is important when using these complexes in high salt environments. For instance, highly hydrophobic aromatic functionalized AuNPs have the highest binding constant in salt-free water but are most disturbed by increasing salt levels. This is potentially due to disrupting the weak binding between the π -face of the polymer cationic ligand termini. For better analysis, ligand hydrophobicity determined by partition coefficient was plotted against the obtained binding constant of the complex (Figure 2). In brief, the results showed no increase in binding with increased hydrophobicity in the case of the aliphatic particles. In contrast, the binding constant using aromatic particles was shown to be proportional to the ligand hydrophobicity on the particle, which is to be expected given the aromatic groups on both the polymer and particle. The binding affinity is lowered when increasing the salt level to physiological levels, but with binding constants of 10^6 to 10^7 these complexes are still useful for IDA systems. In general, these studies indicate that by modifying the surface functionality of AuNPs to alter overall hydrophobicity, a large range of binding constants between particle and polymer can be generated to create potential sensor elements.

Detection of Proteins

The change in protein concentration in biological fluids, such as urine or saliva, can be important indicators of a wide range of pathological conditions.[13] Detecting these changes using array-based approaches would provide an encouraging high speed alternative to current methods for protein sensing such as enzyme-labeled immunoassays and electrophoresis methods. As a first example using polymer/NP constructs, Rotello et al. created a sensor array composed of six cationic functionalized AuNPs (1,2,4,6,11, and 15) and the anionic PPE1 that properly identified seven common proteins.[14] As each protein generates a different interaction with the polymer complex, varied fluorescent responses can be obtained, allowing for discrimination. For example, β -galactosidase shows a relatively large change in the fluorescence of the complex due to its large molecular weight and high negative charge ($pI = 4.6$, 540 kDa). Through the use of linear discriminant analysis (LDA), the fluorescent signature of all of the proteins were able to be separated and unknown samples taken from the protein set could be identified with a 95% accuracy.

Discrimination of proteins through these NP/polymer systems is quite powerful, as each functionalized AuNP can have a different response toward a given protein. However, detection of proteins in simple water solutions, while an interesting proof-of-concept, is not

adequate for discrimination of analytes in more clinically relevant biological environment such as serum, sperm, sweat, and urine. Of these fluids, human serum is the most common and useful biological matrix containing more than 20,000 different proteins.[15] Given the large amount of biological species in this matrix, it is a rigorous media to challenge the sensing strategy. To reduce the nonspecific interactions between serum proteins and the fluorophore, green fluorescent protein (GFP) was used, as PPE polymers will show non-specific interactions with the serum proteins, leading to reduced system sensitivity.[16] GFP has the fluorophore core embedded in a barrel-shaped protein, preventing the aggregation-induced quenching and excimer formation often observed with conjugated polymers.[17]

In the clinic, preliminary serum testing is carried out by simple electrophoresis to observe any substantial protein variations arising from liver imbalances and other diseases,[1] while *specific* proteins found in small amounts are typically detected using antibody arrays.[18] Newer methodologies that attempt to incorporate additional methods such as 2 dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-SDS-PAGE)[1] electrophoresis and surface-enhanced laser desorption/ionization (SELDI) mass spectrometric methods[19] have been recently developed. The limitations of each of these systems are quite apparent as while antibodies are quite specific in terms of binding, they require a different antibody for each protein, simple electrophoresis has limitations for what it can detect, while mass spectrometry requires extensive instrumentation and sample throughput is low. Given these deficiencies, an alternative method for determining protein levels in serum would be very attractive to the clinicians. To see if the AuNP/polymer system could function in this role, a collection of positively charged NP (2,4,7,10, and 15) were chosen and combined with the negatively charged GFP in human serum.[20] As twenty proteins make up 99 % of the serum protein content by weight with human serum albumin (HSA) (70 %), immunoglobulin G (IgG) (14 %), transferrin (5.7 %), fibrinogen (2.8 %), and α -antitrypsin (0.7 %) specifically being 93 % of the overall content,[15] Rotello et al. spiked those 5 proteins into the system to determine if the system could detect changes in their levels akin to a disease state present in a patient. A 2:1 ratio of AuNP to GFP was used where 500 nM was the concentration of AuNP, creating an optimum complex for signal generation. Figure 3 shows the fluorescence changes of the NP-GFP complex in serum upon introduction of 500 nm of HSA, IgG, fibrinogen, antitrypsin, and transferrin, respectively. Using LDA as seen before, all but IgG and antitrypsin were easily differentiated in a two dimensional plot, showing no overlap between the defined circles. The research also describes that mixtures of two of the chosen proteins as well as differing concentrations of one protein produced specific and repeatable patterns in this LDA system.

To determine what drives the nanoparticle/polymer interactions and to get the most differentiation of proteins using the minimal number of sensing elements, Rotello and colleagues employed a structure – activity relationship analysis on both components.[21] By using PPE polymers PPE2 and PPE3 that are modified to prevent aggregation and self-quenching,[12] AuNPs were shown to have a greater binding affinity due to the increased surface area on the polymer. More importantly, they investigated the effect of the sensor by changing the physicochemical properties such as hydrogen bonding, π - π stacking, and hydrophobicity on the headgroup of the AuNP. By using a Jackknifed classification matrix (Figure 4), combinations could be obtained that successfully differentiated 12 distinct

proteins in solution. In particular, hydrophobic functionalized AuNPs especially are far better materials for differentiating than aliphatic particles. Overall, this method opens the door to better understand the underlying operation of the sensor for future studies.

It is clear from these results that AuNPs functionalized with simple electrostatic and hydrophobic moieties can successfully differentiate a wide spectrum of clinically relevant proteins. By continuing to explore the interactions between AuNPs and fluorescent moieties, these sensing complexes can be powerful clinical tools for protein analysis in wide range of complex biorelevant solutions.

Bacteria Detection

Detection and quantification of bacteria in water is of critical need for global health. Bacterial contamination of drinking water impacts billions of people worldwide leading to 19 million deaths each year.[22] Furthermore, bacterial contamination can also lead to instances of food poisoning as well as to hospital acquired infections. Currently, plating and culturing methods are in use to identify strains of bacteria clinically.[23] These tests usually require days for proper analysis, while environmental conditions of drinking water can change daily or even hourly. While higher speed analysis tools have been developed such as PCR that can detect a select few microorganisms,[24] a general analysis tool for the bacteria detection would be of immense help clinicians and to food safety environments.

In 2008, Bunz et al. developed a AuNP/polymer complex using three functionalized AuNPs (NP 4, 6, and 11) with hydrophobic head groups to sense bacterial samples in water. [25] Initially, PPE1 was quenched with AuNP to generate a complex having only 10% of the fluorescence of the original polymer. As expected upon incubating the complex with negatively charged bacteria, some of the PPE chains are released, generating a turn on fluorescence response. The raw responses of the change in fluorescence were analyzed against 12 different strains of bacteria (Figure 5a). It is important to note that other functionalized AuNP were tried in the system, including hydrophilic and short chain aliphatic groups; none of these particles showed any significant fluorescence recovery upon incubation. Figure 5b shows an LDA plot of the responses, showing the three particles that could discriminate the 12 different bacteria, in particular the three different *E. coli* strains. Given that the groupings of the *E. coli* species are not clustered together, there must be differences in the surface chemistries of the microorganisms. Furthermore, there was no visible trend of groupings between the Gram positive and Gram negative bacteria strands. To test the validity of the sensor for real world applications, bacteria samples from the training set were tested by the sensor showing a 95% success rate.

While AuNPs can be easily functionalized for these types of sensing strategies, recent work by Zhang and colleagues have used iron oxide nanoparticles for the detection of bacteria. They designed a sensor array containing three quaternized magnetic nanoparticles (q-MNPs) with a fluorescent polymer to identify and quantify bacteria.[26] Similar to systems with AuNPs, the bacterial cell membranes disrupt the q-MNP–fluorescent polymer complex, generating a unique fluorescence response array. The response intensity of the array is dependent on the level of displacement, determined by the relative q-MNP–fluorescent

polymer binding strength and interaction of the bacterial cells with the MNP. To avoid the interference of q-MNP and q-MNP–fluorescent polymer complex that was not bound to the bacteria, the iron oxide particles are removed using magnetic separation, allowing the fluorescence intensity of the supernatant to be measured. The characteristic responses of these solutions show a highly repeatable pattern and can be differentiated by LDA (Figure 6). Based on the array response matrix, this proof of concept approach was used to measure bacteria with an accuracy of 87.5% at 10^7 colony forming units (cfu) per mL.

Both methods described previously to determine bacteria type in solution highlight the power of NP/polymer arrays for simple bacteria detection. For real world applications of this system, it is important to note that drinking and recreational water supplies may contain other contaminants such as high salt concentrations and heavy metals that could alter how the particle and polymer bind together. Overcoming these interferences will allow these NP/polymer assemblies become a low cost method for water analysis.

Mammalian Cell Detection

Given the sensitivity of the sensor system for proteins and bacteria (*vide supra*), differentiating complex biological entities such as mammalian cells could also be explored. For example, changes in cell morphology between a healthy and cancerous cell can be subtle. Determining from a biopsy whether certain cells are potentially cancerous is therefore needed for these types of clinical settings. Cell detection is typically based on protein biomarkers on the cell surface that generally relies on the generation of antibodies.[27] Protein biomarkers inside the cells have been explored by a range of proteomic techniques, e.g. mass spectrometry,[28] and gel electrophoresis (2D-SDS-PAGE).[29] These lysate-based approaches provide a promising strategy for cancer detection, however they necessitate knowledge of cellular biomarkers.

Mammalian cells and their interactions with AuNP/PPE polymer constructs were studied by Rotello et al. in 2009 to differentiate normal cells from their cancerous and metastatic counterparts.[30] Functionalized nanoparticles with cationic surfaces, will interact with membrane proteins, phospholipids, and carbohydrates that are present on the cell surface through electrostatic and hydrophobic interactions (Figure 7a). Three AuNP–PPE constructs (NP6, 11 and 14 with PPE1) differentiated between four different human cancer cell lines. Furthermore the sensor system was also capable of discerning isogenic mammalian cells that are normal, cancerous, or metastatic, e.g. CDBgeo, TD, and V14 with a limit of detection of 20,000 cells (Figure 7b). These overall results not only show the functional power of the AuNP/polymer system to clinically detect and differentiate clinically relevant cells but to understand the fundamental cell surface changes that occur during cancer and other disease states.

Carbohydrate Detection

Carbohydrates are functionally diverse macromolecules that play an intrinsic role in biological functions such as cell-cell recognition, signaling, and microbiological infection. [31] Glycosaminoglycans (GAGs) are an intriguing subset of these polymers that are

comprised of long chain polysaccharides responsible in organisms for molecular recognition, inter- and intracellular signaling, immune defense, and cellular adhesion processes.[32] While these molecules are of key importance as slight molecular changes in GAGs can significantly impact their biological function, detection of these molecules is challenging. For example, the medically relevant GAG heparin is often contaminated with chondroitin sulfate and hyaluronic acid,[33] but analytical methods to test for purity such as mass spectrometry and HPLC require intricate protocols and lengthy analysis times. In a paper published in 2013, Rotello et al. developed an array-based AuNP/PPE3 polymer complex in order to develop a system that could be broadly used to differentiate structurally similar GAGs in solution.[34] In this study, GAGs with small changes in their acetylation and sulfonation while still retaining similar polymeric backbones were chosen to test the overall effectiveness of the system. Through the use of aromatic and sugar functionalized AuNP (NP1,13,15–20), Rotello and colleagues were able to discriminate 11 GAGs with differing chemical signatures using LDA and principal component analysis (PCA). (Figure 8). This report highlights the overwhelming power of these simple NP/polymer systems to detect subtle changes even at the molecular level. These results also indicate that NP/polymer arrays can open new avenues for gaining valuable insight into the supramolecular chemistry of GAGs and their binding with synthetic receptors.

Detection of Pyrophosphates

While AuNPs can be easily synthesized with different core sizes, other metal NPs can be used for sensing of biorelevant molecules. In a recent report by the Bunz group,[35] 10 nm cobalt ferrite spinel nanocubes (CoFe_2O_4)_n were created using dimethylaminobenzoic acid (DMAB) as a stabilizing ligand. By adding PPE2, DMAB is displaced off of the cube, creating a polymer/mixed ferrite particle complex (Figure 9). Significant binding was seen between a 5 μM solution of PPE 2 and a 20 pm solution of nanocubes, where the polymer lost 90 % of its unbounded fluorescence. They hypothesize that the loss of fluorescence is due to FRET as the absorption curve of the nanocubes overlaps with the emission spectra of the polymer.

For sensing purposes, metal oxide surfaces with exposed hydroxyl groups have strong interactions with small hard inorganic oxo anions that could be present in solution. For the created nanocubes, phosphate-based anions had a large effect on restoring the fluorescence of PPE, while most other anions had only slight effect on these constructs. This result is appealing for creating potential diagnostics using these materials as human blood serum has millimolar concentrations of phosphate (Pi) but micromolar concentrations of pyrophosphate (PPi). Small imbalances in the levels of these two anions can be an indicator of numerous cardiovascular and osteoporosis-related diseases. Further investigation showed that the nanocube/polymer complex was turned on by a Pi concentration of 0.2mM while a observable fluorescence change is seen with a PPi concentration of only 2 μM . For more quantitative detection using fluorescence spectroscopy, a concentration of 40 nM PPi can be detected in a 0.1 mM solution of Pi. Overall, this type of proof-of-concept sensor shows promise as a low cost anion medical diagnostic. However, further development is needed in both sensitivity and ion selectivity to tackle challenging biofluids such as urine or serum before being used in a clinical application.

Outlook and Perspectives

Combining functionalized NPs with fluorescent polymers/proteins can give a wide range of self-assembled hydrophobic or electrostatic sensing complexes. Since the driving force behind forming these assemblies is electrostatic in nature, the head group attached to the particle can be altered with a nearly endless amount of different functionality. By incubating these polymer/NP complexes with negatively charged biomolecules or ions such as phosphates or bacteria, the fluorescence of the construct is altered, usually turning on to produce a fluorescence increase. However, in some cases, the fluorescence can be further quenched by the analyte which is possibly due to aggregation of the NP/polymer complex or by having the analyte induce aggregation of the fluorophore.

In summary, this review has shown that these constructs have impressive features for biological sensing. First, these arrays can have high selectivity for a particular analyte when using only three to six nanoparticles. These polymer/NP complexes have been shown to detect and differentiate in water bacteria, proteins and even cell states when probing mammalian cells. When AuNPs are used, very dilute quantities of added proteins can be detected in human serum showing the ability of these systems to work in complex biofluids without losing performance. Most importantly, the sensor elements that drive the array can be altered to look at different analytes by simply changing the generic head group on the particle.

To increase the sensitivity and selectivity of these systems for future research, modifications of these systems needs to be accomplished. For example, more complicated head groups can be incorporated onto particles such as peptides, sugars and even small molecule drug compounds. It should also be possible through the use of synthetic means to alter the binding constants between polymer and particle to change the selectivity and sensitivity towards a particular analyte. One could even envision that with more sophisticated polymer/NP complexes that the sensitivity of the system could rely solely on the amount of fluorophore released. To improve data analysis, using chemometric techniques to analyze the entire produced spectrum instead of a few chosen points may allow improvements in sensitivity, especially when differentiating similar molecules. Finally, these complexes could be incorporated into solid state, microfluidic, or even paper-based diagnostics to produce quick testing platforms for the clinic that do not require complex workups or data analysis.

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Figure 1.

a.) Schematic diagram of nanoparticle-based polymer arrays. Displacement of the quenched polymer by analytes generates different fluorescence patterns for analysis. b.) Fluorophores and NPs described in this review.

Figure 2. Logarithmic relation of binding constants (K_a) and partition coefficient of NP1–NP11. Figure reproduced from Reference 12 with permission from The Royal Society of Chemistry.

Figure 3.

Fluorescence output (a) of five GFP-NP adducts with serum proteins spiked in human serum at 500 nM). b, Canonical LDA score plot for the fluorescence patterns against five protein analytes at a fixed concentration (500 nM) with 95% confidence ellipses. Figure reproduced from Reference 20.

NP1	NP4	NP6	NP11	NP13	NP14	PPE1	PPE2	PPE3
						83	86	74
						79	85	78
						97	89	92
						97	85	89
						97	85	85
						96	92	85
						92	88	94
						85	74	82
						81	76	69
						86	78	88
						89	86	79
						78	76	72
						81	79	81
						99	88	92
						97	90	92
						97	90	82
						97	82	93
						97	89	92
						74	76	72
						86	85	81

Figure 4. Jackknifed classification matrix for identification of proteins using different combinations of 3 nanoparticles in LDA analysis. The table shows that combinations using hydrophobic AuNPs generally have better identification accuracy. Figure reproduced from Reference 21 with permission from The Royal Society of Chemistry.

Figure 5.

a.) Fluorescence responses of specific complexes upon incubation with various strains of bacteria. b.) Canonical LDA score plot of the fluorescence responses. The first two factors consist of 96.2% variance and the 95% confidence ellipse for the individual bacteria are depicted. Figure reproduced from Reference 25.

Figure 6.

Fluorescence response patterns (a) of q-MNP polymer complex in the presence of bacteria (10^7 cfu m L⁻¹), three-dimensional representation of response changes against the three q-MNP polymer system (b) and canonical score plot (c) for response as determined with LDA. The first two factors collate 93.2% of the variance. Each value is an average of six times measurements, and the error bars are shown. Figure reproduced from Reference 26.

Figure 7.

a) Sensing of isogenic healthy and cancerous murine breast tumor cell types using NP-polymer supramolecular complexes.. b) Canonical LDA score plot for the first two factors of the fluorescence response patterns obtained with NP-PPE assembly arrays. Figure reproduced from Reference 30. Copyright 2009 National Academy of Sciences, USA.

Figure 8.

a.) PCA biplot of the 11 GAGs showing a charge-based discrimination (from positive on the left side to negative towards the right with the neutral GAGs in the middle). b.) The Jackknifed classification matrix showing the contribution of each AuNP in the differentiation. Figure reproduced from Reference 34 with permission from The Royal Society of Chemistry.

Figure 9.

Illustration of the cobalt NP/PPE2 construct and fluorescence quenching with conjugated polymers. Displacement of the DMAB by PPE2 is shown. Figure reproduced from Reference 35 34.