Antibody-functionalized polydiacetylene coatings on nanoporous membranes for microorganism detection†

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The preparation and characterization of coatings made from polydiacetylene colloids on nano- and microporous membranes and their potential for the detection of microorganisms are presented.

Polydiacetylene (PDA) is a conjugated polymer, formed through 1,4-addition polymerization of diacetylenes, that has switchable chromism and fluorescence. Numerous studies have utilized the changeable color of the polymer as a signal transduction element in a variety of sensing materials: liposomes,[1] Langmuir–Blodgett (LB) films,[2] and thick films cast from organic solution onto various solid supports.[3] PDA has fluorescence emission maxima around 560 and 640 nm;[4] this fluorescence property has been much less thoroughly explored for sensing applications, but provides a significantly more sensitive response to stimuli.[5]

In addition to its optical properties, the physical form of the PDA also plays an important role in its suitability for particular sensing applications. Solution-based liposomes or colloids are suitable for laboratory assay scenarios where robotic handling is available but are not ideal in the more uncontrolled situations encountered in environmental testing. Solid-state PDA formats are more robust and therefore better suited to the portability and ruggedness desired in environmental testing. Solid-state formats, however, require interaction of the analyte at a solid–liquid or solid–air interface and are limited by diffusion of the analyte to the surface.

Here we present PDA coatings deposited on nanoporous membranes, describe some of their morphological properties, and demonstrate their potential for use in detection. Depositing PDA on nanoporous membranes is a simple and fast method for creating large-area coatings on opaque substrates. The procedure is extremely flexible and can be used to prepare coatings on both free-standing membranes and in 96-well filter plates. Preparation of coatings in a 96-well format allows for a parallel development effort that greatly increases the speed of testing new formulations. Finally, using a membrane as the coating support permits filtration of an analyte solution through the coating and concentration of sufficiently large analytes, such as microorganisms, at the coating surface. This effect should greatly enhance the sensitivity of a sensor as the concentration of the analyte at the interface will help overcome limitations of analyte diffusion.

In general, PDA coatings are prepared by deposition of precursor diacetylene colloids onto nanoporous membranes. These colloids can be prepared from a variety of diacetylene amphiphiles and can contain up to 60 mol% non-diacetylenic amphiphiles (*e.g.* natural or synthetic phospholipids, cholesterol or other steroids, or reactive amphiphiles). These colloids are generally liposomes, but depending on the formulation may have other structures, such as ribbons.[6] The formulations can also incorporate hydrophobic or amphiphilic fluorophores that can participate in resonance energy transfer (RET) with the PDA, increasing the Stoke's shift and the effective quantum yield of the material.[5,7]

The precursor colloids are deposited in 96-well filter plates or onto free-standing membranes. Coatings are deposited onto free-standing membranes *via* positive pressure using a syringe and filter holder. Deposition on membranes in 96-well filter plates is accomplished by negative pressure using a vacuum manifold.

A variety of membrane materials and pore sizes can be used as supports for the coatings, including hydrophilic polyvinylidene difluoride (PVDF), mixed cellulose esters (MCE), cellulose nitrate (CN), Nylon, and polycarbonate (PC). Useful pore sizes range from 50 nm to 450 nm, depending on the precursor formulation and membrane pretreatment. The membranes may be pretreated with a cationic polymer, such as polylysine, to enhance coating formation.

Once the diacetylene coating is deposited it is chilled at 4 °C overnight to encourage organization and is then photopolymerized using 254 nm UV light. This procedure generally results in macroscopically uniform, blue coatings (Fig. 1a and b).

The PDA coatings must be conjugated to specific receptors to be used as sensing materials for the detection of microorganisms. We have focused primarily on antibodies as receptors, conjugating them to the precursor colloids before deposition. Antibodies were prepared for incorporation into precursor colloids by modification with a hydrophobic tail *via* cleaved hinge-thiols or chemically generated surface thiols.[8] We have used phospholipids modified with a maleimide group for reaction with the free thiol generated on the antibody. The hydrophobic-tail-modified antibodies are then inserted into the precursor colloids by detergent dialysis.[9]

Coating thickness, morphology and antibody presence were examined by immuno-TEM. A solution of 10,12-pentacosadiynoic acid (PCDA) liposomes were conjugated to bovine antibodies and deposited on 0.1 μm PVDF free-standing membranes that had been pretreated with polylysine as described above. The bovine antibodies were modified with free thiols by reaction with 2-iminothiolane and then conjugated to an *N*-(6-maleimidohexanoyl)-1,2-distearoyl-*sn*-*glycero*-3-phosphoethanolamine (MX-DSPE) tail before insertion in the liposomes by detergent dialysis. The coatings were polymerized and then fixed in 0.1 M sodium

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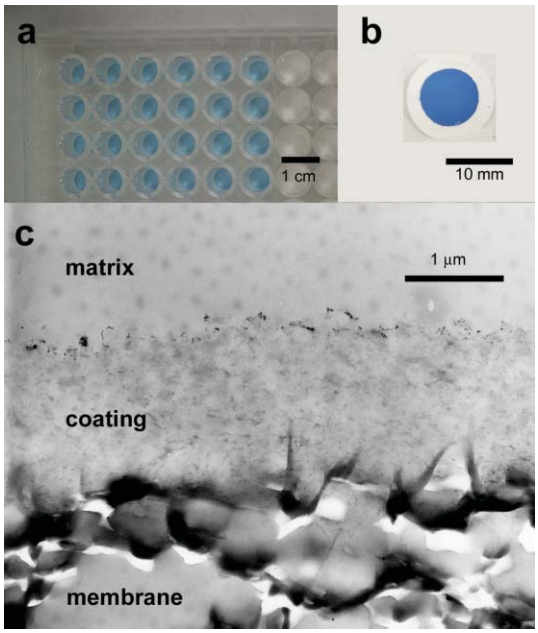

Fig. 1 Images of PDA coatings on nanoporous membranes. (a) PCDA coatings on MCE membranes in a 96-well plate. (b) PCDA coating with conjugated bovine antibodies on a 13 mm PVDF membrane. (c) Immuno-TEM image of a cross-section of (b) with 6 nm gold particles at the coating surface.

phosphate buffer with 4% formalin at pH 7.4. Immuno-TEM observations were performed following the method of Canron *et al*,[10] by Paragon Bioservices, Inc. in Baltimore, MD. Briefly, the coatings were exposed to anti-bovine antibodies labelled with biotin, exposed to streptavidin-labeled 6 nm gold particles, embedded, sectioned, stained and observed. Fig. 1c shows a representative micrograph. The thickness of the coating is about 2 μm, and it has a uniform appearance throughout the entire coating. There are no apparent pores through the coating, even though the membrane pores are visible, and the coating sits on the membrane surface without penetrating into the membrane. Despite the apparent lack of pores, filtering solutions through a coated membrane is nearly as fast as filtering through an uncoated membrane.

Two types of control coatings were also studied by TEM: coatings that contained no antibody and coatings with antibody that were not treated with the secondary antibody but did get exposed to the streptavidin–gold conjugate. Both of the control samples had a similar physical appearance, but neither displayed the gold particles bound to the coating surface seen in Fig. 1c. The particles are clustered in small groups that are spread evenly across the coating surface. The presence of the gold particles demonstrates the presence of the antibodies in the coatings and their accessibility for binding at the surface. The coating preparation method, detergent dialysis insertion of the antibodies into the precursor colloids prior to deposition, most likely results in the presence of antibodies througout the entire thickness of the coating, but interior antibodies are not accessible to the gold particles.

The presence of the antibodies in the coatings was further confirmed by a direct enzyme-linked immunoassay (EIA) using standard procedures[11] (data not shown). EIA was also used to monitor the presence and binding ability of antibodies in coatings stored under humid conditions at 4 °C over the course of more than a year. PCDA coatings were prepared with and without bovine antibodies incorporated *via* detergent dialysis. The bovine antibodies were conjugated to MX-DSPE *via* hinge disulfide reduction or surface-thiols generated from surface amines with 2-iminothiolane. The coatings were deposited in 96-well filter plates with 0.45 μm, polylysine-treated MCE membranes and were stored at 4 °C in a humid environment between assays. Quadruplicate wells were then assayed by EIA weekly for a month, monthly for six more months, and once more after thirteen months. Fig. 2 shows the time course of antibody presentation in arbitrary units[12] for each of the coatings described. There is clearly variation in the amount of antibody present in the coatings depending on the linker and chemistry used. There is also variation in the presentation of the antibodies over time, but on the whole their relative activity remains fairly level, or falls slowly, over the course of the thirteen months.

The coatings also demonstrate good fluorescent stability. Coatings of 10,12-tricosadiynoic acid (TRCDA) were prepared on untreated MCE, CN, Nylon, and PC membranes and the emission was monitored over the course of a year. The coatings were stored at room temperature in the dark between measurements. They were excited at 470 nm and the emission monitored at 642 nm. Fig. 3 shows the time course of the emission on each membrane type. TRCDA on MCE and PC membranes has a very low and stable emission over the whole time course, whereas the coatings on CN and Nylon membranes show significant and nearly continuous increases in emission. It is clear from these results that the choice of membrane is an extremely important factor in designing these materials and therefore we have focused on preparing coatings on MCE and PC membranes to obtain fluorescently stable materials.

To demonstrate the potential of these materials for the detection of microorganisms, we prepared PCDA liposomes with 200 : 1 fluorophore $DiIC_{18}(5)$ for RET amplification of the signal.[7] These liposomes were conjugated *via* detergent dialysis to goat anti-*E. coli* modified with MX-DSPE through the hinge thiols. The

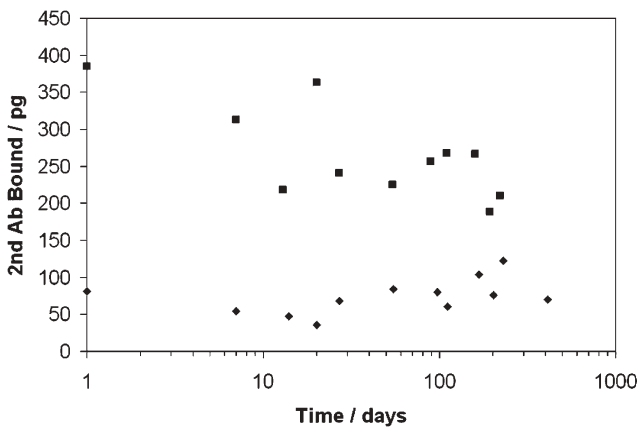

Fig. 2 Antibody presentation over time (log scale) for PCDA coatings conjugated to bovine antibodies and deposited onto MCE membranes in a 96-well filter plate. In both cases the antibodies have a MX-DSPE tail conjugated *via* maleimide/thiol chemistry. In the first case (◆), the tail is conjugated to reduced hinge thiols. In the second case (■), the tail is conjugated to surface thiols generated from reaction with 2-iminothiolane.

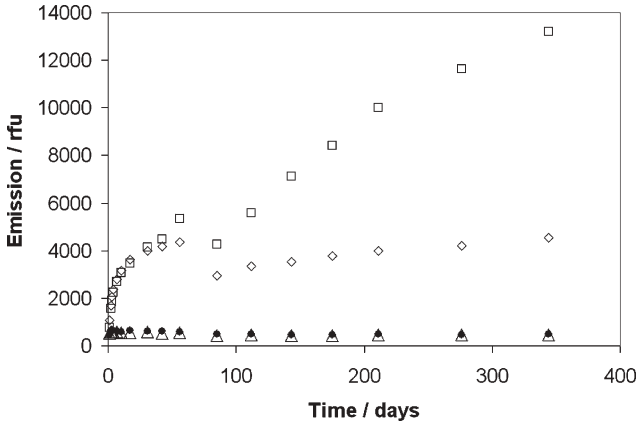

Fig. 3 Fluorescence emission stability over time of TRCDA coatings on four different membrane types: MCE (•), CN (◇), Nylon (□), and PC (△). The membranes were stored at room temperature in the dark.

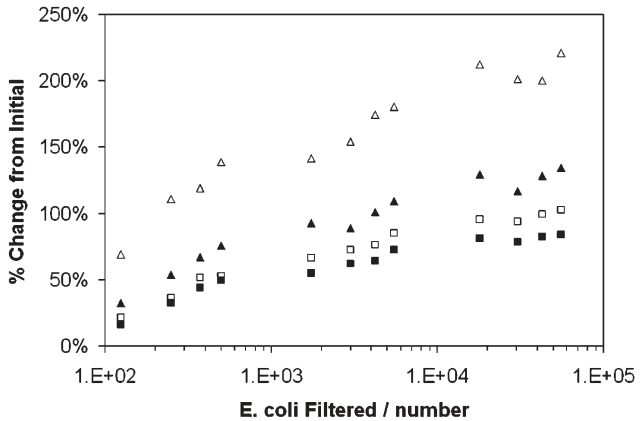

Fig. 4 Change in emission *versus* filtrations (log scale) at 675 nm with PCDA coatings conjugated to goat anti-*E. coli* on MCE membranes when exposed to blank solutions (■,□) or increasing numbers of *E. coli* (▲, △).

liposomes were stored at 4 °C for two and a half months. They were deposited (150 μL per well) in four wells of a 0.45 μm MCE filter plate and photopolymerized. The initial coating emission was measured, with excitation at 470 nm and measurement of the emission at 675 nm. Solutions of *E. coli* were added to two wells and blank solutions to the other two wells. The solutions were filtered through the coatings and the emission was read again. This process was repeated iteratively with increasing concentrations of *E. coli* in the first two wells and blank solutions in the other two wells. Four iterations were done with 125 organisms, four iterations with 1250 organisms, and four iterations with 12 500 organisms per well. Fig. 4 shows the change in emission with number of filtered *E. coli*. The two blank wells show a fairly significant increase in the emission of the coatings, most likely due to the stress of repeated filtration. The two wells exposed to *E. coli*, however, show a larger increase than the blank wells, even at the lowest number of organisms tested (10^2). The response levels off at around 10^4 organisms, which may be due to saturation of the available antibodies.

In conclusion, we have prepared a new material for sensing and assay applications based on coatings of self-assembled polydiacetylene colloids. The colloids make 2 μm coatings on the surface of the membrane and antibodies incorporated into the coatings retain their binding capability for over a year. Additionally, coatings on appropriate membranes are fluorescently stable at room temperature for a similar period of time. Finally, we have demonstrated the ability of these functionalized coatings to respond to the presence of an analyte. In ongoing work, we are assessing the selectivity of the coating response by exposing them to other vegetative-state bacteria and potential interferents.

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Notes and references

1 S. Okada, S. Peng, W. Spevak and D. H. Charych, *Acc. Chem. Res.*, 1998, **31**, 229–239; R. Jelinek and S. Kolusheva, *Biotechnol. Adv.*, 2001, **19**, 109–118.
2 C. W. Yuan, C. L. Lu, Y. Liang, N. Gu and Y. Wei, *Solid State Commun.*, 1991, **80**, 493–495; Q. Cheng and R. C. Stevens, *Adv. Mater.*, 1997, **9**, 481–483; E. Geiger, P. Hug and B. A. Keller, *Macromol. Chem. Phys.*, 2002, **203**, 2422–2431; B. L. Ma, Y. Fan, L. G. Zhang, X. G. Kong, Y. J. Li and J. H. Li, *Colloids Surf., B*, 2003, **27**, 209–213.
3 R. Wang, C. Brown, B. Liu and H. Ribi, *Clin. Chem.*, 1994, **40**, 662.
4 J. I. Olmsted and M. Strand, *J. Phys. Chem.*, 1983, **87**, 4790–4792.
5 US Pat., 6 984 528, 2000. PCT International, WO/00171317, 2001.
6 J. M. Schnur, B. R. Ratna, J. V. Selinger, A. Singh, G. Jyothi and K. R. K. Easwaran, *Science*, 1994, **264**, 945–947; G. Wang and R. I. Hollingsworth, *Langmuir*, 1999, **15**, 6135–6138; Q. Cheng, M. Yamamoto and R. C. Stevens, *Langmuir*, 2000, **16**, 5333–5342; S. B. Lee, R. Koepsel, D. B. Stolz, H. E. Warriner and A. J. Russell, *J. Am. Chem. Soc.*, 2004, **126**, 13400–13405.
7 M. A. Reppy, *Mater. Res. Soc. Symp. Proc.*, 2002, **723**, O5_9_1–O5_9_6.
8 G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, Inc., San Diego, 1996.
9 A. Huang, L. Huang and S. J. Kennel, *J. Biol. Chem.*, 1980, **255**, 8015–8018.
10 M.-H. Canron, S. Bouillot, A. Favereaux, K. G. Petry and A. Vital, *J. Peripher. Nerv. Syst.*, 2003, **8**, 1–7.
11 J. E. Butler, *Methods*, 2000, **22**, 4–23.
12 Units are based on a calibration plot of known amounts of secondary antibody–enzyme conjugate in solution. The kinetics of the solution and interfacial reactions are most likely different, so absolute values cannot be reported.

