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TUTORIAL REVIEW

Using photo-initiated polymerization reactions to detect molecular recognition

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Widely used medical diagnostic devices and assays that sense the presence of a particular molecule in a bodily fluid often rely on either a nanoparticle label or an enzymatic reaction to generate a signal that is easily detectable. In many cases, it is desirable if the magnitude of the signal correlates with the concentration of the molecule of interest. Photo-initiated polymerization reactions are an alternative means of generating amplified signals that can be used to quantify biological molecules in complex fluids. In this case, the formation of a polymer, typically a cross-linked hydrogel, signifies the presence of the molecule of interest. This tutorial review explains how photo-initiated polymerization reactions have been used in a conditional manner to detect and quantify molecular recognition events. We weigh the advantages and disadvantages of using photo-initiated reactions in comparison with other approaches and highlight exciting directions and opportunities in this area.

Learning Objectives

1. Photo-initiated polymerization reactions provide signal amplification that aids in the detection of molecular recognition events.
2. The choice of a reactive system and conditions (initiators, how initiators are coupled to binding events, monomers, dose and wavelength of light) can be used to tune performance.
3. A number of strategies for detecting and interpreting polymerization responses have been investigated and each offers unique advantages and drawbacks.
4. Photo-initiated polymerization reactions and other polymerization chemistries differ in terms of performance and several factors may drive the selection of one approach versus another.

Introduction

Biosensors are molecular sensors that couple biological recognition events with some form of signal transduction. The biorecognition event consists of using a capture molecule such as a nucleic acid sequence or protein to specifically bind a target analyte. In some cases, this interaction is transduced directly into a signal that can be monitored in real time (Figure 1A). Direct, or label-free, transduction occurs in biosensors employing surface plasmon resonance (SPR), in which binding at an interface changes its refractive index and consequently the incident resonant angle at which surface plasmons are excited by plane polarized light.[1] In other cases, following capture of the target analyte by the capture molecule, a second molecule specific to the target and to which a label has been attached is introduced (Figure 1B). Fluorophores and gold nanoparticles are commonly used labels and can be measured directly with fluorescence and absorbance spectrometers, respectively.

Alternatively, the label can serve as the basis of amplification Figure 1C. The enzyme-linked immunosorbent assay (ELISA) employs an enzyme as the label; following the binding steps, a substrate is introduced that the enzyme converts to a colored, fluorescent, or electrochemiluminescent product. Amplification occurs as a result of the ability of one molecule of enzyme to catalytically convert many molecules of substrate to product. Gold nanoparticles and silver salt solutions can also be used for amplification; the silver coats the gold surfaces, catalyzing the deposition of additional silver.

Recently, polymerization reactions performed *in situ* by the user have been investigated as an alternative means of aiding in the detection and quantification of molecular recognition events. In this approach, a radical polymerization reaction amplifies the signal from a molecular recognition event. Typically, initiating molecules are conjugated to a protein or DNA probe that specifically recognizes surface-bound proteins or DNA. This allows for the localization of the reaction to only those regions where the initiating molecule has been immobilized as a function of molecular recognition events (Figure 2). Amplification is inherent to radical polymerization; in response to heat, light, or catalytic activation, each initiator molecule sets off a series of propagation events that result in the conversion of many molecules of a monomer to a polymer. Polymerization-based signal amplification has been implemented with a number of different radical polymerization chemistries, including atom-transfer radical polymerization (ATRP), photo-initiated polymerization, reversible addition-fragmentation chain transfer polymerization (RAFT), and enzyme-mediated redox polymerization. ATRP based methods have been reviewed recently[2]

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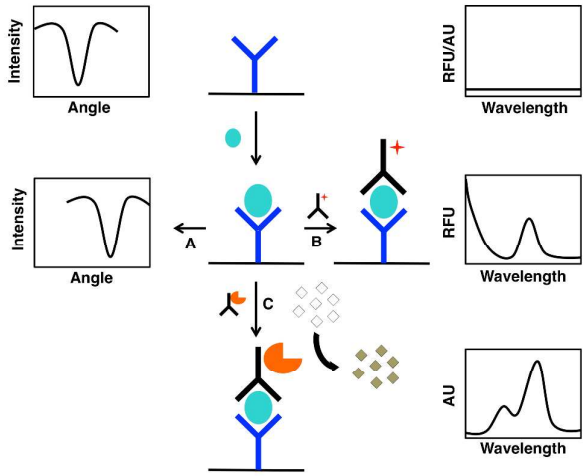

Fig. 1 Biosensing formats depicted for antigen-antibody recognition. A) Binding of the target antigen (circle) to a capture antibody (Y) immobilized on the surface is transduced directly (e.g. surface plasmon resonance). B) A second antibody conjugated to a label (e.g. fluorophore) binds to the target antigen, producing a signal. C) The label conjugated to the second antibody serves as the basis for amplification, converting a substrate into a detectable product that can be quantitatively measured using absorbance (as shown) or fluorescence spectroscopy.

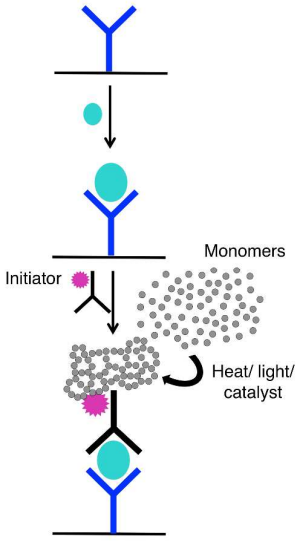

Fig. 2 Polymerization-based signal amplification for antigen-antibody recognition. Following antigen binding to an immobilized capture antibody, a second initiator-conjugated antibody is introduced. This second antibody links polymer formation with capture of the target antigen. Polymerization occurs upon the addition of a solution of monomers and initiator activation in the form of heat, light, or a catalyst.

and photopolymerization as a biosensing tool has been discussed, although not comprehensively;[3,4] here, we consider the development of photopolymerization-based signal amplification (PBA). We discuss the transition from UV light-activated to visible light-activated initiators, introducing the concept of the macrophotoinitiator. We then present key features and design criteria, describing how the response can be tuned by altering the intensity/duration of the irradiation, the motivation behind different monomer compositions, and how to interpret the resulting signal. Finally, we present recent advances and discuss the relative merits of all of the radical polymerization chemistries used for polymerization-based signal amplification.

Development of polymerization-based amplification

Proof of concept

Successful implementation of photopolymerization-based signal amplification hinges on the ability to localize photoinitiators at the site of molecular recognition. This is accomplished through the use of macrophotoinitiators. Macrophotoinitiator is a term that has been adopted to describe a dual-functional molecule comprised of photoinitiators and an affinity ligand. In their simplest form, these

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Professor Hadley D. Sikes leads a team of researchers in the application of physical principles to design, synthesize, characterize and test molecules for utility in detecting and understanding disease. Hadley earned a BS degree at Tulane University, a PhD degree at Stanford University, and was a postdoctoral scholar at the University of Colorado, Boulder, and the California Institute of Technology prior to joining the faculty at MIT. There, she has been honoured with the Joseph R. Mares endowed chair and a Career Award at the Scientific Interface from the Burroughs Welcome Fund.

molecules consist of proteins to which the photoinitiator has been directly conjugated.

In photoinitiated polymerizations, light absorption produces radicals through the excitation of a photoinitiator that then either undergoes bond cleavage to produce radicals (type I) or interacts with a second component through an energy transfer or redox reaction to yield radicals (type II). These radicals then react with carbon-carbon double bonds of monomers in solution, initiating propagation.

In the first demonstration of photopolymerization-based signal amplification, Sikes et al. prepared a macrophotoinitiator by coupling a protein for molecular recognition and UV-activated type I initiators (Irgacure 2959) to poly (acrylic acid co-acrylamide). Upon irradiation with UV light, Irgacure 2959 is cleaved into substituent radical species (Figure 3). In this case, the molecular recognition event was between neutravidin coupled to the polymer backbone and biotinylated oligonucleotides covalently bound to a thin-film biosensor surface.[5] The high affinity (K_d~10^{-15} M) biotin-neutravidin (or alternatively, streptavidin) interaction has been employed extensively in PBA;[5–12] the selection of these binding partners allows the investigation of a limit where the amplification (or signal transduction) step rather than the molecular recognition event determines the limit of detection. The specificity of the binding interaction between biotin and streptavidin results in the localization of initiators at the surface, and this highest known affinity maximizes the number of complexes formed and, consequently, the initiator surface density.

In this first implementation, the monomer solution consisted of 97 wt% hydroxyethylacrylate and 3 wt% ethyleneglycol dimethacrylate. A 10-minute dose of 5 mW/cm^2, 365 nm light produced polymer films that were readily visible using the unaided eye, obviating the need for the optical properties of the biosensor surface.[5] A reliable yes/no response for as few as 1000 molecular recognition events or ~0.005 biotinylated oligonucleotides/μm^2 (based on a 600 μm diameter spot) was achieved. Quantitative information about the number of binding events was revealed by the time when the spots first became visible, with a larger number of binding events resulting in a faster polymerization.

In an effort to expand the applicability of the approach to clinically-relevant antigen detection, Sikes et al. subtyped influenza viruses from crude lysates.[13] In this work, the enzymatic amplification used in a commercially available flu test was replaced with photopolymerization-based signal amplification. The authors used the same thin film biosensor and UV-activated macrophotoinitiator as in the proof-of-concept study,[5] with the exception that the neutravidin was replaced by monoclonal flu antibodies, and showed that photopolymerization-based signal amplification was capable of achieving the same limit of detection as enzyme-based amplification. The assay was designed so that each test contained a built-in positive control (the innermost circle where flu nucleoproteins were covalently bound to the surface as shown in Figure 4). Surrounding the control was the test region where subtype specific monoclonal antibodies were bound, while the remainder of the area served as a negative control. In the absence of virus, polymer was only observed within the positive control spots (Figure 4A). The results when either Flu A or B were present are shown in 4B and C, respectively. The unambiguous response was one of the strengths of PBA relative to enzymatic amplification in this study.[13]

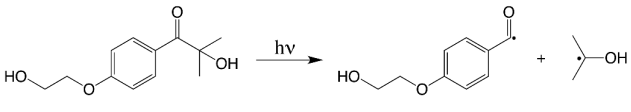

Fig. 3 UV-activated cleavage of Iragacure 2959

Extension to visible light activation

While the initial development work was conducted using a photoinitiator that undergoes bond cleavage in response to UV light activation, an alternative two-component (type II) system has since been used more widely. Building on work in which surfaces were covalently modified with eosin, a xanthene dye, in order to grow hydrogels from these surfaces[14] (i.e. binding events were not involved), photopolymerization-based signal amplification was extended to visible light photoinitiators by coupling the same reaction to binding events.[6] Eosin is a photoinitiator that, following excitation with green light, can undergo a transition to its triplet state, in which it is reactive towards reducing agents such as tertiary amines (Figure 5). An electron transfer from the tertiary amine to eosin in its triplet excited state is followed by a proton transfer that generates two neutral radicals, of which the tertiary amine radical initiates polymerization by reacting with a carbon-carbon double bond of one of the monomers in solution. The neutral eosin radical (the semiquinone form of eosin) is posited to participate in primary radical termination reactions,[15] but is not expected to function efficiently as an initiator due to steric hindrance and electronic delocalization.[16]

Replacing Irgacure 2959 with eosin simplified the macrophotoinitiator synthesis; a commercially available isothiocyanate modified eosin was reacted with the lysine residues of streptavidin (Figure 6A), resulting in an average of two eosin labels per protein. The preparation of the Irgacure 2959 macrophotoinitiator required activation of the carboxyl groups of the poly (acrylic acid co-acrylamide) using carbodiimide chemistry prior to reaction with Irgacure 2959 and neutravidin to form ester and amide linkages, respectively (Figure 6B). Irgacure 2959's low molar absorptivity meant that it was necessary for each macrophotoinitiator to contain 140 Irgacure 2959 initiators in order to induce interfacial polymerization of hydroxyethylacrylate. Direct

Fig. 4 Subtyping influenza viruses with photopolymerization-based signal amplification. (a) No virus detected so polymer is only observed within the positive control spots. (b) Influenza A detected. (c) Influenza B detected. Reprinted with permission from Sikes et al., *Lab on a Chip*, 2009, 9, 633-656. Copyright © 2009, The Royal Society of Chemistry.[13]

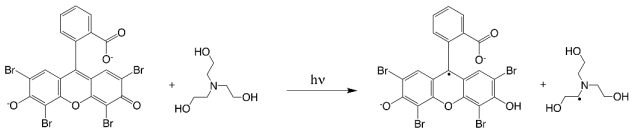

Fig. 5 Visible-light activated energy transfer between eosin and triethanolamine. Eosin is excited by green light to its singlet state, from which it undergoes intersystem crossing to its triplet state. In its triplet state, it is reduced by triethanolamine. A subsequent proton transfer generates two neutral radials. The triethanolamine radical initiates polymerization.

conjugation of Irgacure 2959 molecules to neutravidin produced conjugates that were not capable of initiating interfacial polymerization following binding reactions.

In addition, the assay cost was reduced in the second system by replacing the thin-film biosensor surfaces used originally with glass modified with silanes bearing amino and aldehyde groups. Previous attempts to perform the polymerization reaction on glass substrates using the Irgacure macrophotoinitiators yielded poorly adherent films. The second initiation system was also demonstrated to be compatible with a less toxic, aqueous monomer solution consisting of a tertiary amine coinitiator (typically methyldiethanolamine (MDEA) or trithanolamine (TEA)) and poly $(ethyleneglycol)_{575}$ diacrylate (PEGDA) and 1-vinyl-2-pyrrolidone (VP).

Streptavidin-eosin has become the most extensively used macrophotoinitiator, permitting the detection of unlabeled target analytes using biotin-labeled DNA[17] and biotin-labeled antibodies as detection reagents.[18,19] Eosin has also been directly conjugated to antibodies without loss of protein activity.[20,21] Polymeric macrophotoinitiators, such as those used in UV-activated PBA, remain of interest as vehicles for increasing the number of initiators per binding event. Using fluorescein in place of eosin, Lee et al. demonstrated how the degree of polymerization increases as the number of photoinitiators per binding event is increased.[9] Eosin is the tetrabromo- derivative of fluorescein (Figure 7) and a much

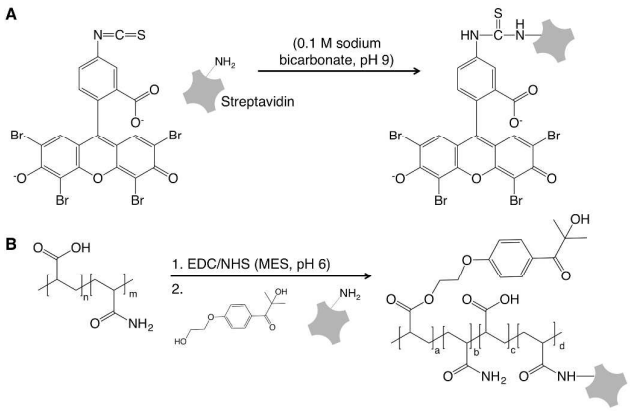

Fig. 6 Macrophotoinitiator synthesis. A) Isothiocyanate-modified eosin reacts with the lysine groups of streptavidin. B) The carboxyl groups of poly (acrylic acid co-acrylamide) are activated by EDC/NHS for reaction with Irgacure 2959 and streptavidin (or neutravidin) to form ester and amide linkages, respectively.

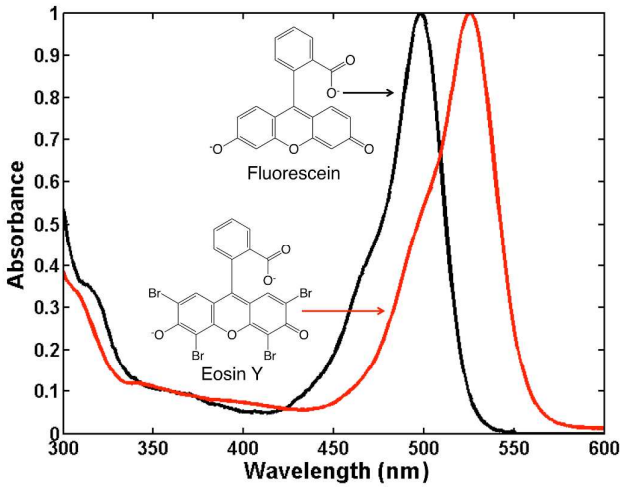

Fig. 7 Structures and absorbance spectra of fluorescein and eosin Y.

more efficient photoinitiator as, due to the presence of heavy bromine atoms, it more readily undergoes intersystem crossing to the triplet state. However, efforts to improve sensitivity through the construction of polymeric macrophotoinitiators incorporating eosin have been stymied by poor solubility[11] and quenching effects.[12]

Johnson et al. explored an alternative method for immobilizing eosin at the site of molecular recognition, essentially constructing a macrophotoinitiator *in situ* for the detection of single base mutations associated with cancer. In this case, surface-bound, synthetic oligonucleotide capture probes hybridized target sequences; these target sequences were designed to emulate human genomic regions with cancer-associated point mutations. Following hybridization of a nucleic acid target with the probe, they used primer extension labeling with eosin labeled 2'-deoxyuridine 5'-triphosphate (dUTP) to facilitate the covalent incorporation of the initiator into surface-immobilized DNA sequences (Figure 8).[22] In primer extension labeling, deoxynucleotides are coupled to the DNA hybrids by a DNA polymerase enzyme. Primer extension labeling with photopolymerization-based signal amplification was previously demonstrated using biotinylated deoxynucleotides (dNTPs),[17] but the procedure developed by Johnson et al. reduced

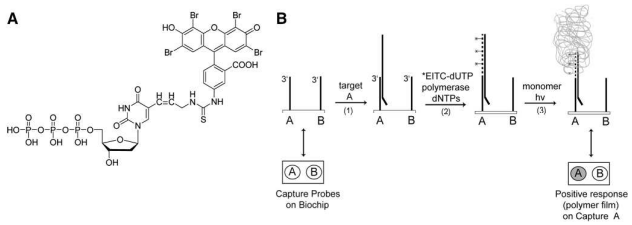

Fig. 8 Macrophotoinitiator synthesis in situ. A) Structure of eosin-modified 2'-deoxyuridine-5'-triphosphate. B) Scheme for detection of nucleic acid hybridization. Capture probe A hybridizes the complementary sequence, target A. Primer extension labeling is used to couple the photoinitiator, eosin, to the hybridized DNA. This is followed by photopolymerization-based signal amplificiation, indicating that target A has been detected. Reprinted with permission from Johnson et al., *Biomacromolecules*, 2010, 11, 1133-1138. Copyright © 2010 American Chemical Society.[22]

the number of required binding steps by eliminating the incubation with streptavidin-eosin.

Design features and criteria

Light activation and the tunable threshold

One of the central features of photopolymerization-based signal amplification is the ability to tune the intensity and duration of the irradiation in order to eliminate signal from nonspecific binding and thereby limit the occurrence of false positives. As depicted schematically in Figure 9, PBA is a threshold process; a minimum surface density of initiators is required for polymerization. Shorter reaction times and lower light intensities can be used to shift the amplification threshold, so that polymerization is only observed for initiator densities above the level of nonspecific binding.

Light source selection is primarily guided by the absorbance spectrum of the photoinitiator (Figure 10), while the duration of the irradiation is generally selected as the time beyond which there are no further gains in sensitivity. For Irgacure 2959 initiated polymerizations, UV lamps were used to supply 10 minute doses of 5 mW/cm^2 light centered around 365 nm.[5,13] With the shift to eosin-initiated reactions, high pressure mercury lamps with filters to restrict the range of irradiation wavelengths to 495-650 nm were used to deliver 20-30 minute doses of 10-40 mW/cm^2 light. The advent of inexpensive light emitting diodes (LEDs) has permitted a transition from high pressure mercury lamps to high intensity LEDs.[20] In addition to being less expensive than mercury lamps, LEDs have the advantage of relatively narrow bandwidths (5-20 nm). This means that the full intensity of the light source can be centered around the maximum absorbance wavelength of the initiator. Relative to mercury lamps, LEDs are also more efficient, both in terms of cost and energy usage, safer, and more environmentally friendly.

Monomer composition

The criteria for monomer selection have evolved since the first demonstration of PBA. Initially, monomer compatibility with the initiating system and the formation of a polymer film were sufficient. As efforts to use PBA quantitatively and translate the technology to the point-of-care have intensified, the criteria have become more stringent.

For the Irgacure 2959 initiated system, the monomer solution consisted of 97 wt% hydroxyethylacrylate and 3 wt% ethyleneglycol dimethacrylate (Figure 11). [5,13] These monomers when used in conjunction with the Irgacure macrophotoinitiator provided exceptional sensitivity, generating polymers in response to 0.005 binding events/μm^2. However, this approach, in addition to requiring the use of toxic, non-aqueous monomers, produced visible yet mechanically unstable polymer films, precluding further analysis or processing steps.

With the transition to the eosin-initiated system, it became possible to employ an aqueous monomer solution consisting of 225 mM methyldiethanolamine (MDEA), 435 mM polyethyleneglycol diacrylate (PEGDA) (M_n 575) , and 37 mM 1-vinyl-2-pyrrolidone (VP) (Figure 11).[6] PEGDA was included as a cross-linking agent and 1-vinyl-2-pyrrolidone (VP) as an accelerant.[23] MDEA has been used interchangeably with triethanolamine (TEA), a more water-soluble tertiary amine. This monomer formulation, also used by Kizilel et al.,[14] was based on work from the Hubbell Group, in which porcine islets were encapsulated in hydrogels through the nonspecific adsorption of eosin onto the islet surfaces.[24] A critical advantage of this chemistry is that the attachment of the polymer to the surface is robust, a feature attributed to termination reactions involving the surface immobilized eosin molecules.[14] This system was shown to be approximately 1000x less sensitive in comparison to the UV light initiated system. However, the advantages, including a simplification of the macrophotoinitiator synthesis, use of less toxic, aqueous monomer solutions, and compatibility with visible light

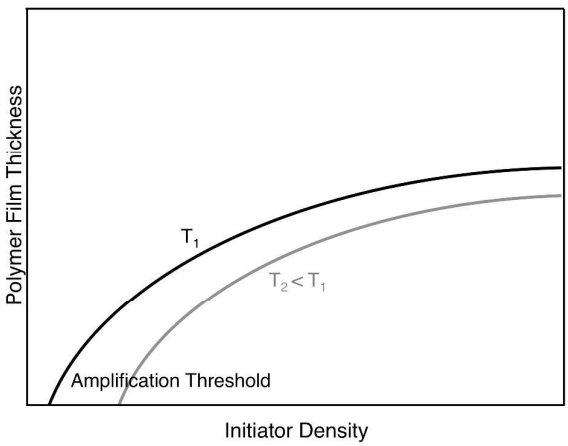

Fig. 9 Amplification threshold. A minimum initiator surface density is required to initiate polymerization. Decreasing the reaction time increases the minimum initiator surface density, shifting the amplification threshold to the right.

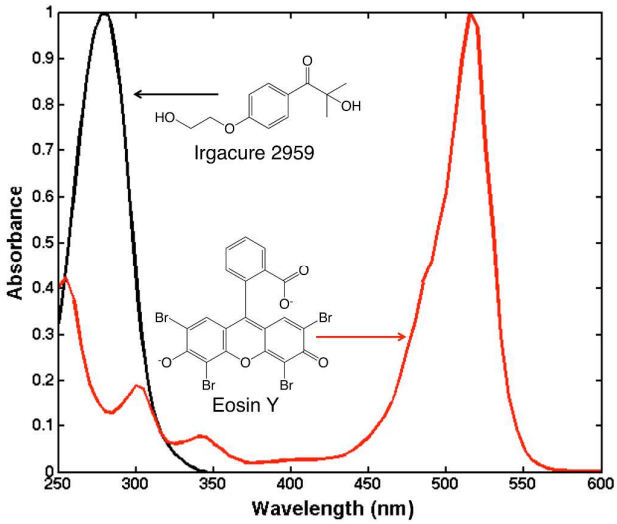

Fig. 10 Absorbance spectra of Irgacure 2959 and Eosin.

and glass surfaces, have balanced the trade-off in sensitivity.

In an effort to optimize the composition of the monomer solution and improve the sensitivity of the signal amplification reaction, Avens et al. tested different formulations of MDEA and VP with either PEGDA or acrylamide/bisacrylamide (Figure 11). They compared polymer film thickness, conversion as measured by real-time IR spectroscopy, and the lowest initiator density for which polymerization was observed (or sensitivity).[25] For PBA, thick polymer films are desired as they are more easily visualized. Thicker polymer films correlate with higher conversion. IR spectroscopy is used to monitor the progression of polymerization reactions by measuring absorption arising from the vinyl groups of the monomer. Total monomer conversion is taken as the fractional change in the initial area of the absorption peak due to an acrylate C-H vibration that occurs in the near IR.

Varying the monomer composition, Avens et al. found that increasing the PEGDA concentration resulted in thicker films, but reduced sensitivity, likely a consequence of the reduced diffusivity of the amine coinitiator in the much more viscous monomer solution. They also tested the hypothesis that streptavidin rearranges in nonaqueous monomer formulations, causing eosin to become sterically inaccessible. Replacing eosin with a streptavidin-eosin conjugate in a bulk monomer solution completely inhibited the polymerization in the nonaqueous formulation. Although biocompatibility is an important consideration for end use at the point-of-care, it is notable that the Irgacure system, for which a nonaqueous monomer solution was used, had exceptional sensitivity.

The tertiary amine coinitiator (MDEA or TEA) also functions as a chain transfer agent, terminating propagating polymer chains and initiating the formation of new ones. This enhances film growth at an optimal concentration but can lead to increased termination and reduced sensitivity if the concentration is too high.[6] Compared with the PEGDA-based formulation, the acrylamide-based monomer (40 wt% acrylamide) produced films that were at least 4x thicker and improved the sensitivity even further, reducing the threshold surface density to 2.8 eosin/μm^2.[6] This was attributed to acrylamide's lower molecular weight and the lower crosslinking content (bisacrylamide) of this monomer formulation promoting faster surface-mediated polymerization by allowing MDEA to more readily diffuse.[6]

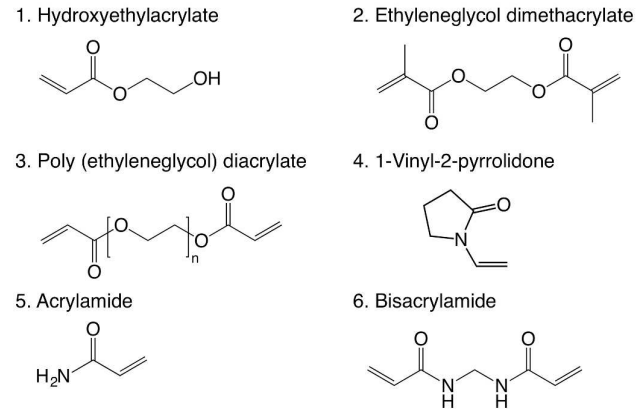

Fig. 11 Monomers for photopolymerization-based signal amplification.

In spite of the advantages of using acrylamide, aqueous, PEGDA-based monomer formulations have ultimately been favored for their lower toxicity. In addition, the PEGDA-based monomer formulation was found to perform better for applications requiring high spatial resolution, such as immunostaining; the smaller average mesh size of the polymers formed using PEGDA allows for the entrapment of a higher density of nanoparticles in a thinner film.[8]

Signal interpretation

The polymer films generated using PBA are generally visible with the unaided eye. Profilometry and fluorescence have been explored as modalities for enabling analyte quantification. This section will include a brief discussion of each readout method.

Visible

The first studies using the Irgacure 2959 macrophotoinitiator and nonaqueous monomer formulation yielded reliable yes/no responses, requiring no instrumentation to detect polymer formation.[5,13] This was shown in Figure 4 for influenza subtyping with PBA.

Profilometry

The transition to the two-component system with eosin and a tertiary amine coinitiator enabled post-processing steps such as profilometry to measure the thickness of the polymer films.[6] The thickness of hydrogels polymerized using a 20-minute dose of 8 mW/cm^2, 400-500 nm light ranged between 110 and 160 nm for 100 binding events/μm^2. A quantitative relationship between polymer film thickness and probe density was later established by Hansen et al.; profilometry revealed a dynamic range between 60 and 4,700 biotin/μm^2 with saturation beyond 8,300 biotin/μm^2 and a maximum polymer thickness of 250 nm.[7]

Fluorescence

As an alternative to the expensive methods available for measuring the thickness of nanometer scale films, strategies for polymerizing fluorescent films were tested to enable quantification using fluorescence signals. Fluorescent moieties were incorporated into the monomer solution to generate an amplified, fluorescent signal that could be directly correlated with the surface probe density.[7,18] Polymer films were made fluorescent by the inclusion of fluorescent nanoparticles (initially, 20 nm Nile Red Fluospheres) in the monomer solution or post-polymerization modification of remaining pendant double bonds with dithiothreitol for reaction with methacryloxyethyl thiocarbamoyl rhodamine B, a fluorescent dye.[7] The latter method proved insufficient for the purposes of quantification, while the addition of fluorescent nanoparticles into the PEGDA-based monomer solution reduced the polymerization kinetics and the final conversion, a possible indication of light attenuation. Hansen et al. also showed that at longer reaction times (>40 minutes), the Nile Red Fluospheres were capable of initiating polymerization. The enhanced sensitivity and thicker polymer films

achieved using an acrylamide-based formulation[25] mitigated some of the detrimental effects of incorporating the fluorescent nanoparticles into the PEGDA-based monomer formulation; their inclusion in an acrylamide-based monomer solution allowed for an improvement in sensitivity relative to direct fluorescence labeling.[18]

Using an acrylamide formulation containing yellow/green nanoparticles, Avens & Bowman compared fluorescent polymerization-based signal amplification (FPBA) with direct fluorescence labeling using either a streptavidin-fluorescein conjugate or streptavidin-functionalized yellow/green nanoparticles on antibody microarrays. They showed that FPBA reduced the limit of detection by more than two orders of magnitude (to 0.16 + 0.01 biotinylated antibody/μm^2) relative to streptavidin-fluorescein, while no positive signal was obtained using the streptavidin-functionalized nanoparticles.[18] In addition, FPBA had a wide dynamic range of 4 orders of magnitude compared with a dynamic range spanning only 2 orders of magnitude for streptavidin-fluorescein labeling. As in the earlier study, there were indications of light attenuation by the nanoparticles. The inclusion of nanoparticles resulted in thinner films, but did not compromise sensitivity. Monomers covalently attached to fluorophores were considered as an alternative strategy for creating fluorescent polymer films; however, a considerable amount of photobleaching and nonspecific polymerization were encountered.

Avens et al. demonstrated FPBA as a nonenzymatic signal amplification technique for immunostaining.[19] Eosin had previously been nonspecifically adsorbed onto the surfaces of porcine islets for the interfacial polymerization of immunoprotective hydrogels.[24] Using FPBA, the fluorescent signal generated is comparable to tyramide signal amplification (TSA) with the advantage of not being affected by endogenous peroxidase enzymes. FPBA also appears to give better signal localization than TSA. The authors successfully labeled nuclear pore complex proteins, vimentin, and von Willebrand factor in fixed and permeabilized human endothelial colony-forming cells, also showing multicolor immunostaining of multiple antigens through sequential polymerizations using differently colored nanoparticles (Figure 12). In the case of the nuclear pore complex staining, FPBA was shown to reduce nonspecific signal by 5.5 fold relative to TSA.

Colorimetric

Although its quantitative potential is more limited relative to profilometry and fluorescence, colorimetric detection is preferred in some point-of-care settings, as it does not require expensive equipment for the detection of polymer films. Kuck and Taylor introduced an additional assay step in which the polymer film is stained with a solution of eosin Y for the purposes of visualization, showing the potential of photopolymerizaton-based signal amplification as an inexpensive colorimetric assay and eliminating the need for profilometers or fluorescence scanners and microscopes (Figure 13).[20] Kuck and Taylor's colorimetric assay has since been demonstrated for the detection of S. pyogenes, which causes group A streptococcal infections,[26] and in the context of E. coli strain genotyping.[27] As a stain, eosin Y outperforms hematoxylin, which requires a longer staining step and yields lower contrast.[17] Using eosin as a stain, Lee et al. showed that colorimetric intensity correlates with polymer film thickness (Figure 14).[9]

The immunostaining technique first demonstrated by Avens et al. using FPBA was further developed to allow for colorimetric labeling of cells, termed "Polymer Dye Labeling" (PDL).[28] Here, eosin Y was replaced as a stain with Evans Blue as eosin nonspecifically stains cytoplasmic proteins and collagen. Like FBPA, PDL has sufficient resolution to enable the identification of protein expression spatial patterns in cells. The authors showed that the signal is stable for over 200 days (Figure 15). Both PDL and FPBA are reported to offer better site specificity than competing enzymatic methods. PDL was originally developed as an alternative to FPBA that is compatible with mounting media, although the authors showed that the samples fared well under dry storage as well. For PDL, the concentration of the tertiary amine was reduced by a factor of 10 relative to the FPBA monomer formulation, with TEA being used in place of MDEA. This optimized formulation may limit adverse effects from chain transfer reactions. In accordance with the results of Lee et al., the colorimetric intensity of the staining loosely correlated with polymer thickness.

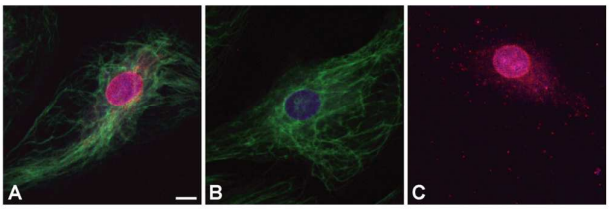

Fig. 12 Immunostaining with sequential rounds of fluorescent polymerization-based signal amplification (FPBA). A) Nuclear pore complex proteins in fixed and permeabilized human endothelial cells were labeled using Nile red nanoparticles; vimentin was labeled in a second round of FPBA using yellow/green nanoparticles. B) Negative control in which the antibody against the nuclear pore complex protein was omitted. As a result, only the vimentin was polymerized. C) Negative control in which the vimentin antibody was omitted and only the nuclear pore complex protein was polymerized. No polymer was produced in the second round. The cell nucleus was also stained with 4', 6-diamido-2-phenylindole dihydrochloride (DAPI; blue). The scale bar is 10 microns. Reprinted with permission from Avens et al., *Journal of Histochemistry and Cytochemistry*, 2011, 59, 76-87. Copyright © 2011, Avens et al.[19]

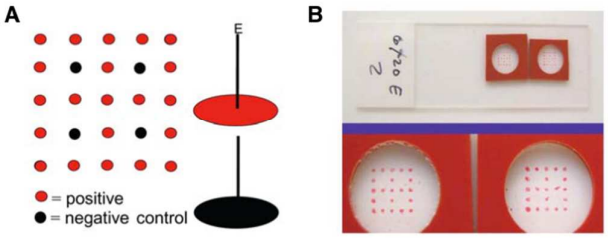

Fig.13 Colorimetric detection. A) Positive control eosin-labeled oligonucleotides were printed in the indicated pattern on an aldehyde functionalized glass slide. Unlabeled oligonucleotides were printed as a negative control. B) The resulting polymers were stained with an Eosin Y solution. Reprinted with permission from Kuck and Taylor, *BioTechniques*, 2008, 45, 179-186. Copyright © 2008, BioTechniques.[20]

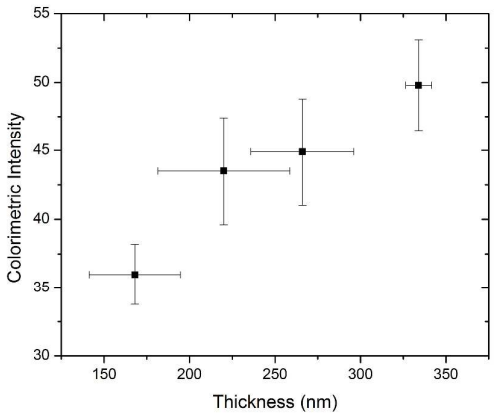

Fig. 14 Colorimetric intensity correlates with polymer film thickness. Average colorimetric intensities were calculated from digital images of polymerized spots. The thickness of each spot was measured using profilometry. Data from Lee et al. *Biomacromolecules*, 2012, 13, 1136-1143.[9]

Addressing practical challenges for use at the point-of-care

Photopolymerization-based signal amplification has the potential to transform diagnostics, both in the laboratory and at the point-of-care. For widespread use, however, several aspects of the reactions and how they were performed and interpreted in initial demonstrations are not ideal. This section will summarize advances relating to oxygen inhibition, instrument-free quantification, and inexpensive surfaces that retain protein structure and function.

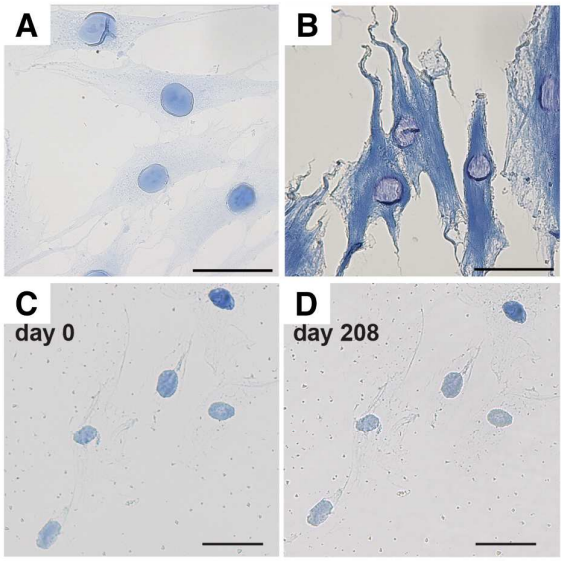

Fig.15 Polymer dye labeling. Evans blue-stained polymers localized in response to labeling with antibodies against nuclear pore complex (A) and vimentin (B). Polymer dye labeling of nuclear pore complex immediately following staining (C) and 208 days later (D). Reprinted with permission from Lilly et al., *PLoS ONE*, 2014, 9, e115630. Copyright © 2014, Lilly et al.[28]

Oxygen inhibition

Radical polymerization reactions are typically inhibited by oxygen.[16] Oxygen reacts with propagating radicals to form peroxy-based radicals that react much more slowly with monomers than propagating radicals and thus have the effect of inhibiting the reaction.

$$R\cdot + O_2 \rightarrow ROO\cdot$$

The dissolved oxygen concentration (0.3-2 mM[15]) is frequently sufficient for complete inhibition. Consequently, it is common to remove oxygen by purging both the monomer solution and the reaction chamber with an inert gas, a practice that increases the cost and complexity of performing these reactions. Nitrogen and argon are most commonly used for this purpose in PBA.

Other strategies for limiting the impact of oxygen inhibition include increasing the light intensity and the photoinitiator concentration.[16] These solutions are impractical for PBA where the initiator concentration is limited by the number of binding events on the surface. This means that increasing the light intensity would likely only deplete the few initiating molecules at a faster rate through reactions with oxygen. Type II systems are reported to be less affected by oxygen inhibition than type I systems as the amine coinitiators can react with peroxy radicals to reinitiate polymerization according to the expression below where DH is the hydrogen donor (the amine).[16] Hydrogens bound to carbons adjacent to an electron-rich heteroatom (oxygen, sulfur, nitrogen) tend to be abstractable.[16]

$$ROO\cdot + DH \rightarrow ROOH + D\cdot$$

Ligon et al. identified the most effective strategies for overcoming oxygen inhibition as those that can either avoid peroxy radical formation entirely or reinitiate polymerization from the peroxy radicals that form. In a mechanistic study of bulk polymerization reactions with eosin, Avens and Bowman found that eosin in conjunction with a tertiary amine coinitiator is able to overcome a 1000-fold excess of oxygen in solution.[15] They proposed a mechanism through which eosin is cyclically regenerated; the peroxy radical abstracts a hydrogen from the semiquinone form of eosin, regenerating eosin in the process and effectively reinitiating polymerization.

$$\text{Initiation: } TEA + E \xrightarrow{h\upsilon} TEA\cdot + EH\cdot$$

$$\text{Regeneration: } ROO\cdot + EH\cdot \rightarrow ROOH + E$$

On the basis of the proposed regeneration mechanism, we showed that the inclusion of submicromolar concentrations of eosin Y in a monomer solution allowed photopolymerization-based signal amplification reactions to proceed under ambient conditions.[10] The addition of 0.3-0.7 μM eosin Y to an aqueous monomer solution containing 200 mM PEGDA, 100 mM VP, and 150 mM TEA allowed polymerization to proceed under ambient conditions only in those areas in which streptavidin-eosin conjugates were bound to biotinylated oligonucleotides covalently coupled to a glass surface. The reaction mixture contained eosin in two forms: dilutely distributed throughout the solution and localized at a higher

concentration at the surface as a function of molecular recognition events. Because the reaction is a threshold process, the polymerization is limited to the surface provided that the local concentration exceeds the minimum initiator threshold where propagation reactions become competitive with inhibition reactions. We showed that the light dose required for the reaction is inversely related to the eosin concentration in the monomer solution; increasing the eosin concentration from 0.3 to 0.7 µM reduced the reaction time required from 100 to 35 seconds without compromising sensitivity. The sensitivity achieved using this system was comparable to that of the purged system; polymerization was initiated from all features with at least 15 molecular recognition events per square micron. However, relative to the purged system for which the thickest polymer film was 0.23 ± 0.02 µm,[7] the hydrogels produced under ambient conditions were considerably thicker with an average maximum height of 0.60 ± 0.27 µm.

Enabling visual quantification

Instrumentation is a common prerequisite for obtaining quantitative information. However, Johnson et al. showed that interfacial features containing differing densities of oligonucleotide probes can be exploited to produce an assay capable of visual quantification of target DNA levels.[22] As shown in Figure 16, higher capture probe densities permit the detection of lower concentrations of target DNA in solution. The number of polymerized spots provides a quantitative estimate for the target DNA concentration in solution. In this work, primer extension was used to covalently couple photo-initiator modified nucleotides to probe sequences that formed DNA hybrids with target sequences while leaving unhybridized probes unmodified.

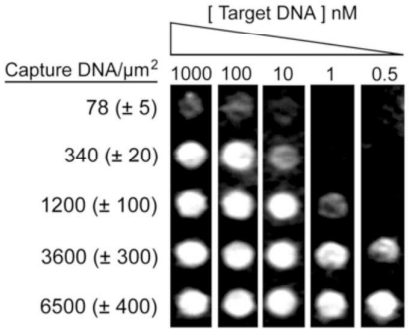

Fig. 16 Visual quantification of target DNA solution concentrations. Biochips printed with capture probe dilution series were separately hybridized with decreasing concentrations of complementary target DNA. The polymers were stained with hematoxylin and the reverse-grayscale images were acquired with a desktop flatbed scanner. Reprinted with permission from Johnson et al., *Biomacromolecules*, 2010, 11, 1133-1138. Copyright © 2010 American Chemical Society.[22]

Inexpensive, biofunctional surfaces

In nearly every microarray implementation of polymerization-based signal amplification, self-assembled monolayer (SAM) modified glass surfaces were used to immobilize capture molecules. In the course of developing an ambient tolerant monomer solution, we found that PBA has sufficient sensitivity to report on surface inconsistencies in SAM coatings.[10] This observation led to the conclusion that a more robust, yet inexpensive, surface was required. In addition, as many molecular diagnostic applications are based on protein binding events, a surface on which protein activity is retained following the surface-immobilization process is required.

We found that coating glass slides with an activated agarose film[29] facilitated a retention of binding activity for proteins that were denatured on SAM-modified glass.[30,31] In addition to providing a more hydrophilic environment, we hypothesize that the agarose coatings shield the proteins from the charged glass surface. These surfaces have the added advantage of being much less expensive than SAM-modified glass from commercial sources (80% lower cost).

The agarose surfaces were critical to the development of a photopolymerization-based epigenotyping assay using methyl binding domain (MBD) proteins.[31] Photopolymerization-based signal amplification enhances the suitability of MBD proteins for the detection of methylated DNA fragments by reducing the cost and complexity of the assay relative to previously proposed methods.

More recently, photopolymerization-based signal amplification was demonstrated as part of a paper-based immunoassay.[21] Photopolymerization-based signal amplification was used to enhance the visual contrast of the colorimetric readout and accelerate the color development process relative to existing enzyme and nanoparticle-based techniques. High visual contrast between negative and positive results was achieved through the formulation of a pH-responsive monomer solution; 1.6 mM phenolphthalein was incorporated into the acrylate monomer solution[10] and the pH was reduced to 7.9 to eliminate light absorption by phenolphthalein during the polymerization. The phenolphthalein becomes entrapped within the polymer network; a basic rinse post-polymerization permits visualization by the unaided eye as the polymer goes from colorless to bright pink. The immunodetection of *Plasmodium falciparum* histidine-rich protein 2 in human serum was used as an example. This method was shown to be quantitative when combined with cellphone-based imaging (Figure 17). Indicative of the flexibility of this assay, it was also possible for the user to decouple the analyte capture from the amplification and visualization; eosin is stable on the surface and is able to initiate polymerization after 130 days of storage, and the hydrogel post-polymerization can be rehydrated with NaOH after 130 days to generate a strong colorimetric response.

Comparison of polymerization-based signal amplification methods

Polymerization-based signal amplification depends on the localization of radical polymerization initiators as a function of molecular recognition events and, thus, is a fairly versatile technique as demonstrated by its implementation with other

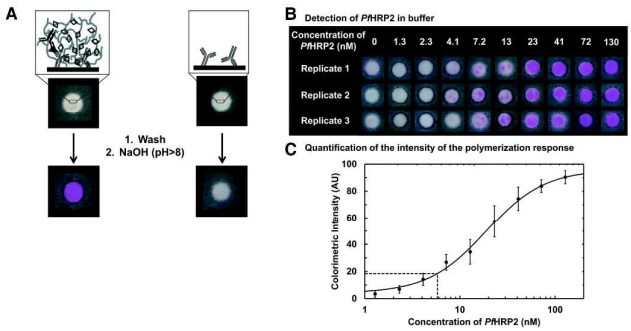

Fig. 17 Photopolymerization-based signal amplification for a paper-based immunoassay. A) Hydrophilic test zones were created by wax printing chromatography paper. Capture antibodies against *Pf*HRP2 were covalently coupled to aldehyde groups within the test zone, allowing for the subsequent capture of *Pf*HRP2. A second eosin-modified reporter antibody was introduced to form a complex with the surface-immobilized *Pf*HRP2 and restrict polymerization to test zones in which *Pf*HRP2 was captured. In order to visualize the polymer that formed, phenolphthalein was included in the monomer solution. As shown, the phenolpthalien became entrapped within the cross-linked polymer network. A colorimetric readout was achieved when a basic solution was dropped onto the surface, causing the phenolphthalein to become pink. A similar color change was not observed on surfaces on which no *Pf*HRP2 was captured and, consequently, no polymer formed. B) Detection of *Pf*HRP2 in a buffered solution at various concentrations. C) Quantitation of the colorimetric results shown in (B). Images were taken using a cell phone camera and the colorimetric intensity was calculated using ImageJ. The vertical line indicates the calculated limit of detection. Reprinted with permission from Badu-Tawiah and Lathwal et al., *Lab on a Chip*, 2015, 15, 655-659. Copyright © 2015, The Royal Society of Chemistry.[21]

radical polymerization chemistries, including ATRP, RAFT, and enzyme-mediated redox polymerization.

Table 1 provides a summary of the molecules coupled to detection probes and the corresponding molecules that initiate radical polymerization by reacting with monomers in solution. The surface immobilized molecules are also highlighted in red in Figure 18. In the two-component initiation system used for photopolymerization-based signal amplification, eosin is attached to the surface, while the tertiary amine radical initiates polymerization (Figure 18A). For the enzyme-mediated method, glucose oxidase is conjugated to avidin and, thus bound to a surface on which biotinylated antibodies are immobilized as a function of molecular recognition events.[32,33] In this case, glucose and iron, two molecules critical to producing initiating radicals, are included in the monomer solution. Rather than functioning primarily as an inhibitor, oxygen is cleverly recast as a participant in the initiation process. The series of reactions resulting in the formation of initiating radicals are summarized in Figure 18B. After first being reduced in the process of converting β-D-glucose to δ-D-gluconolactone, glucose oxidase is regenerated following oxidation by oxygen. The oxidation reaction produces hydrogen peroxide, which is converted to hydroxyl radicals through Fenton's Chemistry. The hydroxyl radicals initiate polymerization by reacting with monomers in solution.

ATRP and RAFT are both examples of living radical polymerizations, in which bimolecular termination reactions are minimized and the lifetimes of living polymers are extended by

Radical generation method	Surface-immobilized molecule	Energy input	Initiating molecule
Photoactivation	Eosin	Light	Tertiary amine radical
Enzyme conversion and Fenton's chemistry	Glucose Oxidase		Hydroxyl radical
Atom transfer radical polymerization	Bromoisobutyrate		Isobutyrate radical
Reversible addition-fragmentation chain-transfer polymerization	Chain transfer agent (CMP)	Heat	2-cyanoprop-2-yl radical

Table 1. Summary of methods of generating initiating radicals

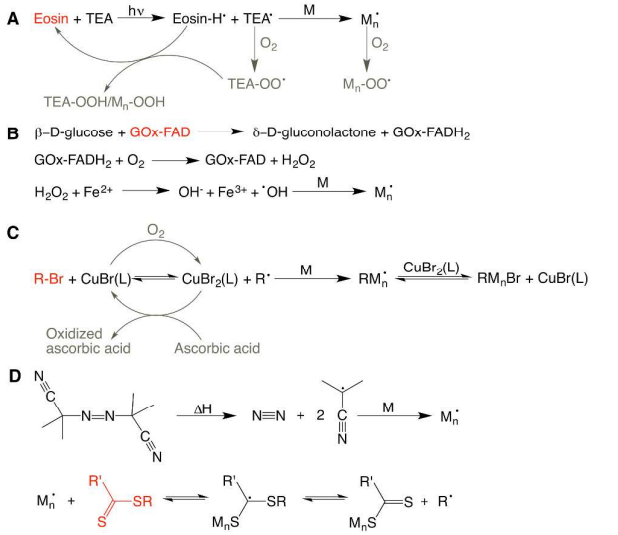

Fig.18 Radical polymerization chemistries for polymerization-based amplification. For each example, the surface immobilized molecule is highlighted in red. A) Photopolymerization using eosin and triethanolamine (TEA) as coinitiators. The TEA radical reacts with monomers (M) in solution to initiate polymerization. Oxygen is an inhibitor, reacting with initiating and propagating radicals to generate peroxy radicals. It has been proposed that the peroxy radicals will abstract a hydrogen from the semiquinone form of eosin (Eosin-H•), regenerating eosin.[15,38] B) Enzyme-mediated redox polymerization. Glucose oxidase is reduced in the process of converting β-D-glucose to δ-D-gluconolactone. Molecular oxygen oxidizes the enzyme, producing hydrogen peroxide, which is converted to hydroxyl radicals by Fenton's Chemistry. These hydroxyl radicals initiate polymerization by reacting with monomers in solution. C) Activators generated by electron transfer for atom transfer radical polymerization (AGET ATRP). In ATRP, a transition metal compound (cuprous halide) catalyzes a reversible redox process involving an organic halide, generating an initiating radical. L is the ligand that complexes the cuprous salt, thereby solubilizing it. $CuBr_2$(L) reduces the propagating radical concentration and minimizes termination.[34] Oxygen inhibits the reaction by oxidizing the cuprous halide. In AGET ATRP, a reducing agent, such as ascorbic acid, is introduced to reduce the Cu(II) complexes to active Cu(I) complexes. D) Reversible addition-fragmentation chain transfer (RAFT) polymerization. A conventional initiator is used to generate radicals in the presence of a chain transfer agent. Heat is used to decompose azobisisobutyronitrile (AIBN) into a nitrogen molecule and two 2-cyanoprop-2-yl radicals that initiate polymerization by reacting with monomers in solution. The dithioester chain transfer agent reversibly transfers a labile end to a propagating chain.[34]

introducing dormant states for the propagating species.[34] When ATRP is implemented for polymerization-based signal amplification, bromoisobutyrate is covalently bound to a detection probe and localized at the surface through binding events.[35] An initiating isobutryate radical is generated when the bromine is transferred to a Cu(I) catalyst in the monomer solution[36] (Figure 18C). The reverse process stops the propagation. With RAFT, a dithioester chain transfer agent is attached to a detection probe and radical initiators, such as azobisisobutyronitrile (AIBN), are included in the monomer solution. 2-cyanoprop-2-yl initiating radicals are formed upon the thermal decomposition of AIBN (Figure 18D). These radicals react with monomers in solution and the propagating chains diffuse to the surface where they then react with chain transfer agents.[37]

With the exception of ATRP, for each of the radical generation methods described, the molecules initiating polymer chains are in solution rather than tethered to the surface. In RAFT, the chain transfer agent at the surface functions to anchor the polymer. It is less clear how this is accomplished for the photopolymerization-based and enzyme-mediated methods, although, in the case of the former, it has been hypothesized that eosin radicals at the surface terminate polymer chains, thereby attaching them to the surface.[14]

As discussed previously, one of the barriers to wider implementation of polymerization-based amplification has been that radical polymerization reactions are inhibited by oxygen. The enzyme-mediated system is unique in that oxygen is required for the production of initiating radicals. We have described how submicromolar concentrations of eosin in a monomer solution allow the eosin-initiated photopolymerization to be performed under ambient conditions.[10]

In addition to quenching propagating radicals, oxygen inhibits ATRP by oxidizing the Cu(I) catalyst.[39] In an approach termed activators generated by electron transfer (AGET), a reducing agent, such as ascorbic acid, is introduced to circumvent oxygen inhibition. The ascorbic acid returns the Cu(II) complexes to their reactive lower oxidative state [39 and references therein]. (Figure 18C)

RAFT was initially explored as an alternative living polymerization method intended to address some of the limitations of ATRP, including nonspecific adsorption of metal ions to DNA molecules, which contributes to elevated background signal, and the use of toxic transition metal catalysts. Although RAFT achieved an order of magnitude increase in polymer thickness (with a maximum of ~200 nm) and lower background relative to ATRP,[37] its susceptibility to oxygen inhibition and heat activation requirement have limited interest in developing the method further. Recently, Chapman et al. demonstrated the potential utility of glucose oxidase for oxygen scrubbing in open vessel RAFT polymerizations.[40] Instead of bubbling argon through the monomer solution for 10 minutes prior to heat activation of the initiator, glucose oxidase is added to the monomer solution and left at room temperature for the 10 minutes preceding activation. Whether this strategy would work for interfacial polymerizations remains to be seen.

Because polymer growth is linear with respect to time, controlled radical polymerization methods such as ATRP and RAFT lend themselves to quantitative detection. AGET ATRP was implemented for quantitative electrochemical biosensing by triggering radical polymerization on an electrode surface.[41] Monomers such as 2-hydroxyethyl methacrylate (HEMA) and glycidyl methacrylate (GMA) were selected to provide excess hydroxyl and epoxy groups, respectively, for the attachment of electrochemical tags (aminoferrocene). The combination of AGET ATRP with electrochemical sensing provided a linear range spanning five orders of magnitude. More recently, Wu et al. successfully implemented AGET ATRP as part of a paper-based microfluidic electrochemical immunodevice for the detection of four cancer biomarkers (Figure 19).[42] Looking to the future, the authors envision a portable, low-cost diagnostic enabled by the rapidly evolving electronics manufacturing landscape.

However, while the electrochemical output simplifies

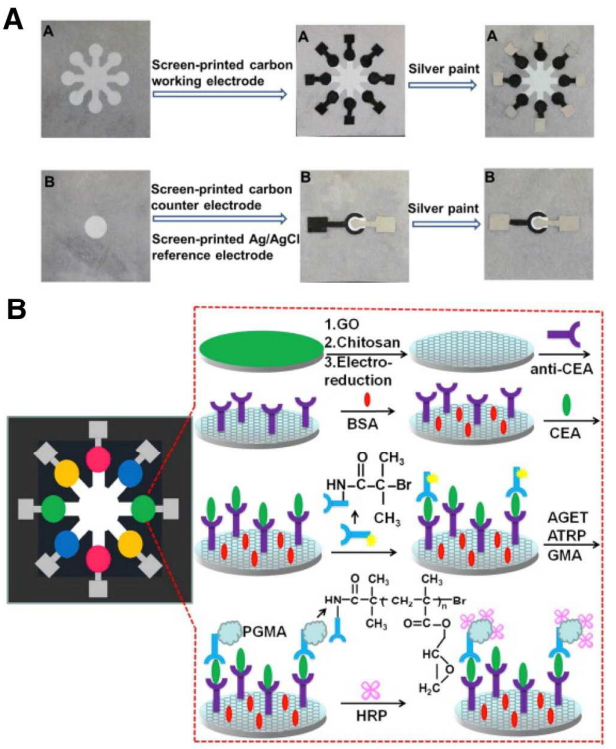

Fig.19 A paper-based electrochemical device integrated with AGET ATRP. A) The device consists of two layers of patterned square filter paper. Layer A consists of a central connecting zone and 8 working zones. Layer B has one connecting zone corresponding to that of layer A. The chromatography paper was impregnated with photoresist and irradiated with UV light to create the patterns. The working zones of layer A were screen-printed with carbon ink. Ag/AgCl and carbon ink were printed on layer B to form the reference and counter electrodes, respectively. B) The device was designed for the detection of four biomarkers: carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), cancer antigen 125 (CA125), and carbohydrate antigen 153 (CA153). The fabrication and assay procedure are shown for CEA. A solution of graphene oxide (GO) was dropped onto each working electrode, followed by a chitosan solution. The GO was then electrochemically reduced and the electrode was incubated with glutaraldehyde. Capture antibodies against each of the cancer biomarkers were applied to the corresponding working electrodes. Remaining active sites were blocked with BSA and a sample solution containing the biomarker (here, CEA) was introduced. The bromoisobutyrate (initiator)-coupled CEA antibody was added and AGET ATRP was performed. A solution of HRP (intended to react with side-chain functional groups of the polymer) was dropped onto each working electrode prior to electrochemical detection. Reprinted with permission from Wu et al., *Biosensors & Bioelectronics*, 2014, 52, 180-187. Copyright © 2014, Elsevier B.V.[42]

quantification relative to ellipsometry (the favored method for measuring the thickness of the films generated using ATRP), the attachment of the electrochemical tags added 10-20 hours to the assay time (on top of 2 hours of AGET ATRP followed by a 2 hour rinse), ultimately limiting its utility. In addition, although AGET ATRP was carried out in the presence of oxygen, the electrochemical experiments were performed under nitrogen,[41] erasing the gains achieved through the use of AGET ATRP. An electrochemiluminescent assay based on ruthenium complex with tertiary amine was also developed for use with AGET ATRP. This method expanded the linear range to seven orders of magnitude and reduced the post-polymerization modification period to 5 hours.[43]

AGET ATRP has been used to enhance the detection sensitivity of surface plasmon resonance (SPR) spectroscopy.[44] Liu & Cheng presented a membrane based biosensing interface using functional gold nanoparticles and AGET ATRP to enable ultrasensitive detection of cholera toxin (LOD=160 aM) by SPR, improving the detection limit by six orders of magnitude relative to direct measurement without amplification. In another membrane application, Liu et al. used AGET ATRP to grow a polymer at the membrane-water interface of a supported lipid bilayer by using initiator labeled trimethylammonium salts to recognize membrane-bound cavitands.[45]

Nanoparticle-based colorimetric methods have been demonstrated using both ATRP[46,47] and enzyme-mediated redox polymerization.[48] In each of these examples, polymerization was used to alter the optical properties of a solution of nanoparticles. The ATRP examples concentrated on the immobilization of initiators on DNA or antibody modified nanoparticles through molecular recognition events, while Gormley et al. used glucose oxidase mediated radical polymerization to entangle negatively charged gold nanoparticles. Glucose oxidase was used to eliminate oxygen from the system as well as generate hydrogen peroxide for the horseradish peroxidase-catalyzed reaction with acetylacetone. In addition to horseradish peroxidase, this concept of nanoparticle aggregation was used for the detection of catalase, iron, and copper.

It is clear that significant progress has been made in the last few years in terms of exploring novel applications, overcoming oxygen inhibition, and enabling quantitation. Each of the initiation chemistries presented has unique advantages and the selection of one amplification method over another must be driven by the end application. For colorimetric, point-of-care assays, photopolymerization is a strong candidate as it is capable of producing thick films that are readily visible to the unaided eye in as little as 35 seconds under ambient conditions. Both of the other oxygen-tolerant methods, AGET ATRP and enzyme-mediated redox polymerization, require hour-scale reaction times and experience sensitivity losses when the reaction time is reduced. The selection of eosin as the photoinitiator also means that inexpensive light emitting diodes can be used for activation. A major advantage of photoinitiated polymerization is the ability to limit the occurrence of false positives by tuning the dose of light, thereby shifting the threshold for the positive response.

Photopolymerization-based signal amplification is limited by the difficulty of accessing a dynamic regime and interpreting the colorimetric readout quantitatively. Johnson et al. presented a possible solution to this problem through careful microarray design,[22] but the wide dynamic range and precision that has been achieved using electrochemical detection with AGET ATRP would be challenging to match. In the published methods, quantification through the generation of an electrochemical output is achieved at the expense of assay time. However, for some applications, the extra time required to generate the final output as well as the reliance on more expensive substrates may be warranted.

Conclusions and Outlook

There are many factors to consider when employing chemical reactions in biosensors, including ease-of-use, expense, time, complexity, and a requirement for instrumentation. In developing photopolymerization-based signal amplification, inexpensive chemistry similar to that employed successfully for adhesives and coatings has been used in a new way to provide an alternative to current amplification methods for point-of-care disease detection. The requirements for free radical photopolymerization chemistry at the point-of-care differ in important ways from the requirements for reactive systems for use in the above mentioned, more established applications. For example, the photoinitiator and monomers should be water soluble, nontoxic, and stable without special storage requirements. Photoinitiators should have reactive functional groups to enable coupling to biorecognition molecules to yield detection reagents. Many strategies that have been developed to circumvent the problem of oxygen inhibition for more established applications of photopolymerization chemistry are either infeasible or problematic for signal amplification. Since initiator concentrations are controlled by the number of binding events, which in turn is limited by the concentration of an analyte molecule that is present in a sample, overcoming oxygen inhibition by using initiator concentrations that are higher than that of oxygen (sub-mM to mM) is not feasible. Purging with inert gases is possible, though not convenient. Step-growth, thiol-ene polymerizations prevent discrimination between interfacial and solution-phase polymerization reactions, an essential capability for connecting the reaction to whether molecular recognition events have occurred. Identification of additional initiation systems that address these challenges presented by biosensing applications is an exciting current direction.

As noted throughout, the visible light-activated system based on eosin and a tertiary amine coinitiator provides a number of advantages in comparison with the original UV-activated system used for photopolymerization-based signal amplification. However, many questions remain about the mechanism through which eosin is able to overcome inhibition of the polymerization reaction by oxygen. In addition, the parameter space of possible chemical reactants has not been fully explored; understanding the mechanism of the current system will likely enable rational, focused exploration of other reactions. This is an area of active research and the mechanism is systematically being elucidated through spectroscopic experiments and kinetic modeling.[49] These efforts will allow for more rapid screening of chemical reactants and contribute to a predictive model that could reduce reliance on trial and error experimentation.

We envision using the most rapid, oxygen tolerant photopolymerization reaction identified to date in a device that can be used to screen for infectious diseases, analogous to those that have been recently field-tested for assessing liver function.[50] Using a polymerization reaction rather than an enzymatic reaction for signal amplification has the potential to address two areas for improvement identified over the course of this field-testing. Specifically, these reactions offer the ability to easily distinguish positive and negative results through improved colorimetric contrast, while also providing flexibility through the automation of reaction times. Testing the reactions in the hands of intended users is an important next step.

This tutorial review has described the development of photopolymerization-based signal amplification as a biosensing technique and how performance using this approach differs from that obtained using other radical polymerization chemistries for signal amplification. Recent advances, including but not limited to the ability to perform the reactions in air and on paper surfaces, have expanded the realm of potential applications. Many opportunities exist for exploring alternative initiation reactions, monomer combinations, and reaction conditions. Investigation of new chemical systems as well as new strategies for detecting the hydrogels that form are promising future directions for expanding the prevalence and utility of biomolecular assays.

Acknowledgements

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