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Balancing the initiation and molecular recognition capabilities of eosin macroinitiators of polymerization-based signal amplification reactions

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Coupling polymerization initiators to molecular recognition events provides the ability to amplify these events and detect them using the formation of a crosslinked polymer as an inexpensive readout that is visible to the unaided eye. The eosin-tertiary amine co-initiation system, activated by visible light, has proven utility in this context when an average of three eosin molecules are coupled to a protein detection reagent. The present work addresses the question of how detection sensitivity is impacted when the number of eosin molecules per binding event increases in the range of two to fifteen. Unlike in other initiation systems, a non-monotonic relationship was observed between the number of initiators per binding event and the observed detection sensitivity.
1. Introduction

Rapid detection tests (RDTs) that enable molecular diagnosis and monitoring of disease have had significant impact on human health, and current research efforts focus on increasing the number and types of disease states that may be routinely and reliably monitored in this way.\textsuperscript{1-4} Cost, ease-of-use, and performance are each of major importance for wide adoption of detection platforms and techniques.\textsuperscript{5} With these criteria in mind, we and others have developed polymerization-based signal amplification\textsuperscript{6-9} as a method that provides rapid (< 1 min) and unambiguous visualization of results in RDTs using low-cost chemistry that is currently used in a wide variety of inexpensive consumer products\textsuperscript{10-12}. In polymerization-based amplification (PBA), initiators of radical polymerization reactions are localized as a function of molecular recognition events, and upon activation using light, a cross-linked hydrogel forms from an aqueous monomer solution. The resulting micron-scale hydrogel\textsuperscript{7, 9} is easily visualized without instrumentation, and the technique has been used to detect DNA hybridization\textsuperscript{8, 13, 14}, antibody-antigen recognition\textsuperscript{15-17}, and other protein-protein binding events\textsuperscript{18}. PBA tolerates complex sample matrices including serum\textsuperscript{13}, cell lysates\textsuperscript{9}, and fixed and permeabilized cells.\textsuperscript{17}

To date, several polymerization initiators have been investigated for use in PBA.\textsuperscript{6, 7, 19} Of these, the eosin/tertiary amine co-initiation system provides the best performance in an oxygen environment in terms of detection sensitivity accompanied by a rapid reaction rate (< 1 min).\textsuperscript{8, 9, 19} The limit of detection occurs when the local initiator density in the vicinity of binding events of interest falls below a threshold level required for the polymerization reaction to overcome inhibition by molecular oxygen. With an average of three eosin molecules coupled to each binding event, detection limits have been established in several test systems.\textsuperscript{19, 13, 18} In the present work, we investigate whether increasing the number of eosin molecules localized per binding event improves the detection limit, as expected on the basis of our previous observations in the fluorescein/tertiary amine co-initiation system.\textsuperscript{19}
end, we have synthesized a set of dual-functional polymers, termed macroinitiators, bearing a protein for molecular recognition and a variable number of eosin substituents for the ability to couple initiation of radical polymerization to molecular recognition.

2. Results and Discussion

Scheme 1 shows the syntheses of amine-terminated eosin 1 and macroinitiators 6–9, and a conceptual representation of PBA for colorimetric detection. The aminated eosin was synthesized from eosin 5-isothiocyanate (EITC) through thiourea bond formation. Briefly, tert-butoxycarbonyl (Boc)-protected amine was employed here to prevent the formation of undesired products, such as dimer eosin. After the formation of a thiourea bond, cleavage of the Boc group using TFA\textsuperscript{20} afforded aminated eosin 1, which was tethered to poly(acrylamide-co-acrylic acid) by an amide coupling reaction (see Supporting Information). Eosin-functionalized copolymers 2–5 were purified by dialysis, precipitation, and size exclusion chromatography and subsequently conjugated with neutravidin; as a result, macroinitiators 6–9 were obtained (see Supporting Information). As depicted in Scheme 1B, macroinitiators were used to connect molecular recognition with a polymeric readout. Neutravidin, incorporated in the macroinitiator, recognized biotin-labeled DNA coupled to a glass surface. Upon exposure to visible light in an air environment, the eosin macroinitiators reacted with tertiary amine co-initiators to initiate a polymerization of acrylate monomers. The resulting hydrogel was stained with a dye for colorimetric read-out as previously reported.\textsuperscript{19}

Eosin-functionalized copolymers 2–5 were characterized by $^1$H NMR and UV–visible spectroscopy. In the proton NMR spectra with D$_2$O as a solvent (Figure 1), the x-axis values were chosen to highlight the range of chemical shifts displayed by the aromatic protons of eosin, and the peaks were assigned by $^1$H NMR measurement of free EITC in deuterated
DMSO. We monitored a noticeable peak-broadening trend as a function of reaction stoichiometry (Figure 1a–d). In the other words, as the molar ratio of eosin to the copolymer increased in the reaction mixture, the spectral features arising from the eosin moiety broadened. For example, while the spectra of 2 and 3 had several characteristic peaks in the range of 7.2 to 8.0 ppm (Figure 1a–b), these features broaden markedly and shift slightly to lower ppm for compounds 4 and 5 (Figure 1c–d). Although the broadness hindered a determination of the number of eosin molecules per copolymer for all cases, the integration results support the increasing trend for yield as a function of stoichiometry, where the protons of the copolymer backbone were used as a reference peak at 1.2–2.2 ppm. As a complementary characterization strategy that we have successfully used in past work,[19] UV–visible spectroscopy was employed to determine the average number of eosin per copolymer. This analysis yielded the following average numbers of eosin per copolymer: 2 for 2, 6 for 3, 10 for 4, and 15 for 5.

After neutravidin was coupled to the eosin/copolymers 2–5 via an amide bond, macroinitiators 6–9 were obtained with yields > 70% as measured by the HABA (4’-hydroxyazobenen-2-carboxylic acid) assay.[19] The macroinitiators were tested for specific binding capabilities using glass surfaces covalently modified with replicate spots of biotin-labeled DNA at 5 different surface densities (Figure 2a). Detailed information on the test surface, including density of biotin and spot size, was obtained by fluorescence techniques as previously described.[9,19] Figure 2b–d shows fluorescence responses resulting from the incubation of 6–9 with the test surface. Interestingly, macroinitiator 8 generates higher fluorescence intensity in the target spots than the other macroinitiators; for example, the average intensities were 20800 (± 1100) for 6, 34000 (± 1100) for 7 (see Figure S1), 52400 (± 240) for 8, and 30600 (± 580) for 9 in the first row (Figure 2b–d). On the other hand, the fluorescence intensity of the background is proportional to the number of eosin molecules per the copolymer, where the average intensities were 1450 (± 460) for 6, 2420 (± 680) for 7 (see
Figure S1), 25900 (± 3100) for 8, and 26800 (± 350) for 9. In the case of macronitiator 9, a slight increase in background intensity (i.e. nonspecific binding) was accompanied by a dramatic decrease in the average spot intensity (i.e. specific binding) compared with the results using 8 (Figure 2e).

The finding that an average of 15 initiators per binding event leads to a lower surface-localized signal than an average of 10 initiators per binding event is contrary to results obtained in past work with other initiation systems, namely fluorescein/tertiary amine and Irgacure 2959.\textsuperscript{[6,19]} In these previous systems, increasing the number of initiators per binding event led to higher surface-localized initiator density as a function of specific binding interactions. While it was possible to synthesize macroinitiators that coupled >100 fluorescein molecules to each binding event, 15 was the observed limit for eosin, a tetrabromo derivative of fluorescein. The aqueous solubility of eosin macroinitiators exhibited a strong inverse proportionality to the number of hydrophobic bromo groups per copolymer. We hypothesize that poor solubility not only interferes with the specific binding of the eosin macroinitiator to the biotin-DNA functionalized surface, but it also increases nonspecific adsorption to the surface in regions that have not been modified with biotinylated DNA. Efforts to optimize the response from molecular recognition using pH did not yield substantial improvements. Because the eosin moiety is known to exist in its dianionic form above pH 7\textsuperscript{[21]} and the acrylic acid residues of poly(acrylic acid-co-acrylamide) are also ionizable, macronitiator 8 was treated under alkaline conditions in order to increase solubility. Resulting signal and background intensities at pH 11 were both 2–5 times lower than at pH 7.4 (Figure 3a and b).

\textbf{Figure 4} shows the polymerization response resulting from molecular recognition between 8 and the biotin-functionalized surface at pH 7.4. Interfacial polymerizations coupled to molecular recognition were not observed using macronitiators 6, 7 or 9 with the monomer solution and illumination conditions as previously described.\textsuperscript{[19]} In Figure 4, macronitiator 8
yielded colorimetric responses from the first 3 rows of features with as few as 50 possible molecular recognition events per square micrometer. This finding represents a three-fold higher detection limit than that attainable with an average of three eosin molecules coupled directly to streptavidin. Figure 4 presents the original image (left), the original with a mask for automated image analysis (middle), and the software-calculated mean intensity from the spots in each row (right). As detailed in earlier work\textsuperscript{[9,18]}, interfacial polymerization coupled to binding events occurs only when the density of initiators localized near the surface as a function of binding events exceeds the threshold required to overcome oxygen inhibition of the polymerization. Initiators localized near the surface as a function of either binding events or nonspecific binding result in shorter inhibition times locally in comparison with the longer inhibition times observed in the bulk solution where initiator concentration is lower. With macroinitiators 6 and 7, the initiator densities inside and outside of the biotin-DNA features were below the threshold for amplification and polymerization was not observed in the 10-250 sec range. With macroinitiator 9, the overall initiator density localized on the surface was higher than observed with 6 and 7, but the difference in initiator densities inside the features and outside the features was not great enough to enable a photoreaction time that resulted in polymerization only in regions where molecular recognition occurred. The initiator density localized within the biotin-DNA features was much lower with 9 than with 8. With 9, for short reaction times, no polymerization was observed, and at longer times bulk polymerization occurred.

It is possible that quenching processes may explain the lower fluorescence intensity obtained within the biotin-DNA features using 9 in comparison with 8. Quenching may occur through a variety of mechanisms in which excited fluorophores undergo non-radiative decay. To our knowledge, initiation of polymerization depends only on absorbance and not on emission (See Figure S2 for a detailed mechanism). The fact that interfacial polymerization coupled to binding events was possible using 8 but not 9 supports the idea that reduced binding of 9,
rather than a quenching mechanism, causes the reduced fluorescence signal. Our observations of reduced solubility with increasing eosin substitution of the macroinitiators suggest the possibility that solution phase aggregates may form at the highest degree of eosin substitution in this study. Unfortunately, dynamic light scattering and x-ray techniques that are commonly used to characterize solution phase structure require concentrations in the range of 2-10 mg/mL, and these concentrations are much higher than the µg/mL concentrations relevant for this work.

3. Conclusions

In summary, a limit has been determined in the eosin/tertiary amine initiation system where increasing the number of initiators per macroinitiator beyond ten begins to impede the function of the macroinitiator in the context of PBA. This result contrasts with observations using other initiation systems\[^{19,22-24}\] In our view, even with this limitation, the eosin/tertiary amine initiation system provides superior performance to other alternatives as detailed in Table 1 of \[^{9}\]. For example, other photoinitiation systems such as Irgacure 2959\[^{6}\] and fluorescein/tertiary amine\[^{19}\] are more sensitive to oxygen inhibition than the eosin/tertiary amine system. Initiators of AGET ATRP have been used to detect molecular recognition in air environments, but a tradeoff between reaction time and sensitivity exists with hours required for maximal sensitivity. In addition, AGET ATRP produces thinner films\[^{24}\] that require silicon or gold substrates for visualization and expensive instrumentation for quantitation of analytes. Because of the capabilities of the eosin/tertiary amine initiation system in comparison to alternatives, increasing the number of eosin initiators localized per binding event remains an attractive strategy for improving the detection limit if the problem of aqueous solubility can be addressed in future efforts.

Experimental
Synthesis of 5-(3-(2-aminoethyl)thioureido)-7a-(2’,4’,5’,7’-tetrabromo-6’-hydroxy-3’-oxo-3H-xanthen-9’-yl)benzoic acid 1 (amine-functionalized eosin). N-Boc-1,2-diaminoethane (3.35 mg, 21 µmol) was slowly added into EITC (12.3 mg, 17.4 µmol), dissolved in THF (6 mL) at 0 °C under nitrogen atmosphere. The reaction mixture was allowed to room temperature, and was protected from light by wrapping the flask with aluminum foil for overnight. The resulting mixture was concentrated by rotary evaporator, and the crude product was precipitated with diethyl ether several times. BOC-protected amino eosin was obtained as a red solid (15 mg, 99%). \(^1\)H NMR (400 MHz, MeOD) \(\delta\) 8.03 (s, 1H), 7.81 (d, 1H), 7.43 (s, 2H), 7.27 (s, 1H), 3.77 (br t, 2H), 3.31-3.35 (2H), 1.41 (s, 9H). MS (MALDI-TOF, matrix: 3-hydroxypicolinic acid): m/z 865.08 (cal. 865.78) [M–H]+. To cleavage the Boc group, trifluoroacetic acid (1 mL) was slowly added into the Boc-protected amino eosin (10 mg, 11.5 µmol) dissolved in the mixture of methylene chloride (0.9 mL) and methanol (0.1 mL) at 0 °C under nitrogen atmosphere. The reaction mixture was allowed to room temperature, and was protected from light by wrapping the flask with aluminum foil for 6 hr. The resulting mixture was concentrated by a rotary evaporator, and the crude product was precipitated with diethyl ether several times. Aminated eosin 1 was obtained as a red solid (8 mg, 90%). \(^1\)H NMR (500 MHz, MeOD) \(\delta\) 8.35 (s, 1H), 8.09 (s, 1H), 7.92 (d, 1H), 7.37 (d, 2H), 7.11 (s, 1H), 4.01 (t, 2H), 3.31-3.35 (2H). MS (MALDI-TOF, matrix: 3-hydroxypicolinic acid): m/z 764.51 (cal. 764.74) [M]+.

Materials and additional details of procedures used herein that were largely established in previous studies are provided as Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library.
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Keywords: photopolymerization, interfacial polymerization, macroinitiator, colorimetric bioassay

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**Scheme 1.** (a) Syntheses of aminated-eosin 1 and macroinitiators 6-9, and (b) conceptual representation of polymerization-based signal amplification (not to scale).

EDC = N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide hydrochloride; NHSS = N-hydroxy sulfosuccinimide; TFA = trifluoroacetic acid
Figure 1. Zoomed-in $^1$H NMR spectra of eosin-functionalized copolymer (a) 2, (b) 3, (c) 4, and (d) 5 dissolved in D$_2$O (10 mg/mL). The integral values for the indicated peaks are included only to highlight the trend, and the number of eosin per copolymer was determined by UV–visible spectroscopy.
Figure 2. (a) Schematic diagram of a test surface. Fluorescence images resulted from the molecular recognition of macroinitiator (b) 6, (c) 8, and (d) 9 with test surfaces functionalized with biotin-labeled oligonucleotides, and (e) a summary of the quantitative analysis of average fluorescence intensities for each region of the test surface. A non-monotonic dependence of intensity on the average number of eosin substituents per macroinitiator is evident for the 1st-3rd rows.
Figure 3. Fluorescence images of macroinitiator 8 bound to the test surfaces under different alkaline conditions.

Figure 4. Colorimetric readout obtained using macroinitiator 8 with a photoreaction time of 105 seconds. Interfacial polymerizations as a result of specific binding events were not observed using macroinitiators 6, 7, or 9 with photoreaction times ranging from 10 to 250 seconds.
When polymerization reactions are used to detect molecular recognition events, the initiation step is crucial in determining the detection sensitivity. The present work examines the relationship between the number of eosin co-initiators localized per binding event and the resulting detection sensitivity.

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Supporting Information

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**Materials**

Poly(acrylamide-co-acrylic acid) (MwL 200 kDa, acrylamide: approximately 20 wt %.

Aldrich) N-hydroxysulfosuccinimide (NHSS, Aldrich), 1-ethyl-3-(3-dimethylaminopropyl)-
carbodiimide hydrochloride (EDC, Aldrich), N-Boc-1,2-diaminoethane (Aldrich),
trifluoroacetic acid (99%, Aldrich), methylene chloride (Aldrich), methanol (Aldrich),
dimethyl sulfoxide (Aldrich), deuterated NMR solvents (Aldrich), buffer solutions (Aldrich),
eosin 5-isothiocyanate (EITC, Invitrogen), neutravidin (Thermo Scientific), 10× bovine serum
albumin (BSA, Thermo Scientific), LH-20 Sephadex (GE Healthcare), centrifugal filter
device (molecular weight cutoff: 100 kDa, Millipore), dialysis tubing system (Spectrum),
biotin-functionalized DNA calibration arrays (InDevR) aqueous ampliPHY monomer
solutions (InDevR) were purchased and used without further purification. The enhancer that
accompanies the monomer solution was not used in this work. We obtained MALDI-TOF
mass spectrometry profiles from Bioneer Inc.

**Synthesis of eosin-functionalized poly(acrylamide-co-acrylic acid) derivatives 2–5.**

Poly(acrylamide-co-acrylic acid) (10 mg) was completely dissolved in pH 8.0 buffer solution
(1 mL) at room temperature. EDC (50 equiv for all), NHSS (100 equiv for all), and aminated
eosin 1 (5, 10, 15, and 20 equiv) dissolved in DMSO (0.2 mL) were added to the solution at
room temperature and then stirred at 35 °C for overnight. To increase the yield of the amide-
coupling reaction, additional EDC (50 equiv) was added to the solution. The crude product
was purified by dialysis, size exclusion column chromatography, and precipitation to remove completely any remaining free aminated eosin after reaction. We initially dialyzed the crude product with a 13 kDa molecular weight cutoff membrane tube against a mixture of deionized water and methanol at room temperature. After the dialysis, the resulting solution was concentrated with ethanol by rotary evaporation and then precipitated with a mixture of acetone (6 mL) and ethyl acetate (12 mL) in a vial. Precipitated red solid was dried under vacuum for 1 day.

**Synthesis of macroinitiator 6-9.** EDC (100 equiv) and NHSS (150 equiv) were added into the solution of compound 2–5 (approximately 0.3 mg, 1 equiv) dissolved in pH 8.0 buffer solution (100–150 µL) at room temperature for 10 min. Neutravidin (1 equiv) dissolved in pH 8.0 buffer solution (50 µL) was added into the reaction mixture at room temperature, and the resulting mixture was incubated at 4 °C for overnight. The crude product was filed using a centrifugal filter device (100 kDa molecular weight cutoff) by three times with pH 8.0 buffer solution. The filtered solution was collected, and then used for photopolymerization.

**Photopolymerization.** The solution containing the co-initiator, triethanolamine, and monomers, 1-vinyl-2-pyrrolidinone, and poly(ethylene glycol) diacrylate, was purchased from InDevR, and used without the addition of the supplied enhancer solution. Photopolymerization was carried out with an ampliPHOX™ Reader (InDevR) at room temperature without inert gas purging. For the preparation of sample, we mixed macroinitiator 6–9 with 20 µL of hybridization buffer (2× PBS and 10× Denhardt’s solution) and 20 µL of 1.5% BSA solution. The resulting solution was incubated with the DNA array chip in a humidity chamber for 10 min at room temperature and rinsed with solutions (1× PBS and 0.1 wt% tween 20 in 1× PBS), and finally deionized water. After the remove of excess water by air stream, 40 uL of the monomer solution was placed in contact with the resulting chip, and then irradiated with 400 nm light from an LED source under ambient conditions.
**Fluorescence imaging.** After the DNA array chip was activated by macroinitiator 6-9, fluorescence image was obtained with a DNA microarray scanner (GenePix 4000B Microarray scanner, Molecular Devices, LLC.), where we used only the green channel with 100% PMT, and the range of intensity was 0–60000 for all images.

![Fluorescent images](image)

*Figure S1.* Fluorescent images of macroinitiator 7 bound to test surfaces after the different concentrations of BSA were pre-treated onto the chip surfaces: (a) 1.5% BSA (40 µL) and (b) 0.5% BSA (40 µL).
Figure S2. A current proposal for the mechanism by which eosin and tertiary amines initiate radical polymerization of acrylate monomers. (Incorporates findings from D. C. Neckers et al. *Tetrahedron* 1987, 43, 4507, C. Bowman et al. *J. Poly. Sci. Part A Polym. Chem.* 2009, 22, 6083, and V. H. Pérez-Luna et al. *Langmuir* 2004, 20, 8652)