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Abstract
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Keywords
coco-localization studies, colocalization, conversion rates, equivalent concentrations, fluorescent dyes, free enzyme, horseradish peroxidase, micelles, glucose sensors

Disciplines
Biochemical and Biomolecular Engineering | Biological Engineering | Molecular, Cellular, and Tissue Engineering

Comments

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Block Copolymer-Quantum Dot Micelles for Multienzyme Colocalization

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ABSTRACT: To mimic the structure and functionality of multienzyme complexes, which are widely present in Nature, Pluronic-based micelles were designed to colocalize multiple enzymes. To stabilize the micelles as well as to enable characterization of single enzyme immobilization and multienzyme colocalization by Förster resonance energy transfer (FRET), quantum dots (QDs) were incorporated into the micelles to form Pluronic-QD micelles using a novel microreactor. Model enzymes glucose oxidase (GOX) and horseradish peroxidase (HRP) were respectively labeled with fluorescent dyes. The results indicated that FRET occurred between the QDs and dyes that labeled each type of enzyme in single enzyme immobilization studies as well as between the dyes in colocalization studies. These observations were consistent with increases in micelle size after adsorption of dye-enzymes as verified by dynamic light scattering. In addition, the activity of single enzymes was retained after immobilization. An optimized colocalization process improved the overall conversion rate by approximately 100% compared to equivalent concentrations of free enzymes in solution. This study demonstrates a versatile platform for multienzyme colocalization and an effective strategy to characterize multienzyme immobilization and colocalization, which can be applicable to many other multienzyme systems.

INTRODUCTION

The design and development of nanoscale platforms for colocalizing multiple active enzymes has been extensively studied due to its potential for efficient catalytic mechanisms.1−4 In living cells, multienzyme complexes are composed of individual enzymes in a confined space, where each component enzyme works synergistically by transporting reactive intermediates among active sites rapidly to promote the overall cascaded reaction efficiency.5,6 Such highly concerted mechanisms possess advantages including maintaining high local concentration of the intermediates and reducing diffusion losses during the long transportation, which are especially critical for highly unstable reactive intermediates.4,7 To mimic this process in vitro, researchers have developed various strategies and approaches to spatially colocalize multiple enzymes on carriers to achieve enhancement in reaction kinetics along with the ability to direct the reaction pathway.8−13

In one example, a multiple-enzyme extract adsorbed on chitin exhibited higher stability over a range of temperature and pH than did the soluble multienzyme extract.8 Another example described immobilization of coupled enzymes in different regions of a porous polymer monolith, which spatially defines small reaction areas to separate multiple enzymes in each portion. In this system, the plug flow direction of the substrate solution corresponding to the order of the cascaded reaction was found to maximize the final product yield.9 To overcome the drawbacks of internal diffusion resistance in porous materials, researchers have used nonporous nanoparticle platforms based on gold, silica, and polymers to colocalize multiple enzymes, where the surface of rigid support materials were functionalized with soft enzyme layers.10−13 For example, by mixing separated immobilized single enzymes and cofactors on silica nanoparticles, which was facilitated by Brownian collisions of the nanoparticles, the enzymes and cofactors were recovered by filtration or precipitation.11 In colocalizing sequential multiple enzymes onto the nanoparticles, the order of each enzyme layer adsorbed on the nanoparticles was found to affect the overall product conversion rate; furthermore, colocalizing the enzymes on the same layer showed the highest catalytic kinetic performance.12,13 Previous work from our laboratories demonstrated a sequential approach to colocalize enzymes on multifunctionalized polystyrene nanoparticles that resulted in kinetic performance improvements.14 However, in addition to demonstrating the kinetic benefits of colocalizing multiple enzymes, it is desirable to control and characterize the colocalization of multiple enzymes on the same nanoparticles. In this work, we designed a novel micelle carrier to colocalize multiple enzymes with the ability to characterize enzyme immobilization and control colocalization.

Pluronic triblock copolymers and Pluronic-based amphiphilic pentablock copolymers developed by us have shown great

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Supporting Information

S Supporting Information

FRET between the QDs and dyes that labeled each type of enzyme in single enzyme immobilization studies as well as between the dyes in colocalization studies. These observations were consistent with increases in micelle size after adsorption of dye-enzymes as verified by dynamic light scattering. In addition, the activity of single enzymes was retained after immobilization. An optimized colocalization process improved the overall conversion rate by approximately 100% compared to equivalent concentrations of free enzymes in solution. This study demonstrates a versatile platform for multienzyme colocalization and an effective strategy to characterize multienzyme immobilization and colocalization, which can be applicable to many other multienzyme systems.
potential in biominalization and drug/gene delivery as self-assembling templates and delivery vehicles.\textsuperscript{15--20} The copolymers serve as organic templates to direct the growth of the inorganic nanoscale phase.\textsuperscript{15,18} These polymers have demonstrated excellent compatibility with proteins and the corresponding micelle structures are responsive to temperature and pH.\textsuperscript{21,22} Recent work has showed that the stability of the micelle structure can be enhanced by encapsulation of hydrophobic semiconductor QDs.\textsuperscript{23} QDs have demonstrated great potential for imaging and biosensing due to their unique rapid response, stability, and efficient fluorescence-based features.\textsuperscript{24--27} Paired with suitable dyes, QDs can be used to measure nanoscale distances between molecules using principles of FRET.\textsuperscript{28,29} In this work, we combined the well-known protein compatibility of Pluronic micelles and the sensing attributes of QDs to develop a novel multienzyme colocalization strategy. Here, hydrophobic organic QDs were incorporated into self-assembled amphiphilic Pluronic triblock micelles in aqueous solution using a flash nanoprecipitation process (Figure 1). To demonstrate the feasibility of this nanoscale platform, a model multienzyme system based on GOX and HRP, which is known to exhibit a sequential reaction cascade\textsuperscript{7} to produce resorufin, was studied. Each enzyme was respectively labeled with appropriate fluorescent dyes to exhibit FRET with the QDs when single enzymes were adsorbed on the micelles and with themselves when the enzymes were colocalized (Figure 1). The enzymatic performance of adsorbed enzyme and colocalized multiple enzymes was evaluated by comparing that of the respective free enzymes.

## EXPERIMENTAL SECTION

1. **Chemicals.** Cadmium selenium (CdSe) quantum dots were synthesized as described previously.\textsuperscript{30} Carboxyl reactive Alexa Fluor 594 (AF594, with excitation/emission wavelengths of 594/620 nm) and Alexa Fluor 647 (AF647, with excitation/emission wavelengths of 647/675 nm) fluorescent dyes and Amplex Red were purchased from Invitrogen (Carlsbad, CA). Pluronic F127, GOX (\~200 U/mg, from Aspergillus niger), horseradish peroxidase (HRP) (\~250 U/mg, from horseradish), and resorufin were purchased from Sigma-Aldridge (St. Louis, MO). Tetrahydrofuran (THF), dimethyl sulfate (DMSO), 3\% hydrogen peroxide, sodium chloride, and trisodium phosphate were purchased from Fisher Scientific (Hampton, NH). All of the aqueous solutions were prepared using purified water from Thermo Scientific’s Barnstead Nanopure Ultrapure Water System.

2. **Fabrication of PLQD Micelles.** In this procedure, Pluronic F127 polymers and QDs were predissolved in THF at respective concentrations of 154 mg/mL and 2.3 mg/mL. Next, 2 mL of well-mixed PLQD solution and three equivalent volumes of phosphate buffered saline (PBS, 0.1 M Na\textsubscript{2}PO\textsubscript{4} and 0.15 M NaCl, pH 7.2) were pumped simultaneously into a four-channel microreactor using syringe pumps at a flow rate of 2 mL/min. The organic and aqueous solutions were mixed in the central mixing compartment, where nucleation and growth of the micelles occurred in the presence of the QDs. The solutions exited the reactor and flowed into a beaker containing 2 mL of PBS buffer for quenching. The final product solution contained 20\% THF, 31 mg/mL Pluronic F127 and 0.5 mg/mL QDs, which was stored at 4 °C. The size of the PLQD and the enzyme-PLQD micelles was measured using a Malvern Zetasizer Nano-ZS90 dynamic light scattering (DLS) system using Malvern disposable cuvettes (Malvern Instruments, Southborough, MA). The sample solutions were diluted 2-fold and the experiments were carried out in triplicate with approximately 10 min for each measurement.

3. **Transmission Electron Microscopy.** The QDs and the PLQD micelles were respectively characterized using a Tecnai G2 F20 scanning transmission electron microscope (S/TEM) (FEI) at an operating voltage of 200 kV. A drop of 1 mg/mL QD suspension in 647/675 nm fluorescent dyes (AF594) was separated using a dialysis membrane centrifuge tube at 10,000 rpm for 1 h and continued at 4 °C overnight. The unattached dyes were separated using a dialysis membrane centrifuge tube at 10,000g for 10 min. The conjugated enzyme-dye solution retained in the dialysis tube was separated using a dialysis membrane centrifuge tube at 10,000g for 10 min. The conjugated enzyme-dye solution retained in the dialysis tube.
was collected and diluted to a protein concentration of 1 mg/mL. The enzyme concentration was determined using UV–vis spectroscopy (Cary 50 MPR microplate reader, Varian, USA) following the dye manufacturer’s standard labeling procedure. The resultant product was stored at 4 °C.

5. Adsorption of Single Enzymes onto PLQD Micelles. Initially, centrifuge tubes were pretreated with 1 mg/mL bovine serum albumin (BSA) solution in PBS buffer to prevent nonspecific protein adsorption onto the walls of the tubes. In each reaction tube, appropriate volumes of AF594 labeled GOX or HRP were mixed with 250 μL of stock PLQD micelle solution. PBS buffer was used to bring up the total volume to 500 μL. The reaction was carried out overnight with rotation at 4 °C. The experiments were performed at various enzyme concentrations. In the final solution, the concentrations of GOX-AF594 were 0.1, 0.25, and 0.625 μM, and the corresponding values for AF594-HRP were 0.91 and 2.27 μM.

6. Multienzyme Colocalization on PLQD Micelles. For multienzyme colocalization, GOX and HRP were respectively labeled by AF594 and AF647 dyes that can be paired to exhibit FRET using a protocol described in Invitrogen’s manual (https://products.invitrogen.com/ivgn/product/A20173). To colocalize both enzyme dye conjugates on PLQD micelles, 20 μL of GOX-AF594 conjugates and 125 μL of HRP-AF647 conjugates were added to 250 μL of a PLQD stock solution with PBS buffer to obtain a final solution volume of 500 μL. The reaction solution containing 0.25 μM GOX and 2.73 μM HRP was incubated overnight with rotation at 4 °C.

7. Single Enzyme Adsorption and Multienzyme Colocalization Using FRET. A FRET study of GOX-AF594- or HRP-AF594-PLQD solution was carried out using a dual monochromator spectroluorimeter (Fluoromax-4, Horiba Jobin Yvon, USA) with excitation at 440 nm and slit widths of 4 nm (excitation and emission) with 5-fold dilution. For the GOX-AF594 and HRP-AF647 colocalization on the PLQDs, a wavelength of 594 nm was used to excite the solvents.

8. Enzyme Assays. The individual enzyme and multienzyme assays were performed as described previously,14 where Amplex red is catalyzed by HRP to produce fluorescent resorufin. The change in concentration of resorufin was monitored using a fluorescence microplate reader (SynergyMx, Biotek, Winooski, VT). For HRP, the kinetic reactions were carried out by adding 20 μL of 100 ng/mL HRP-AF594 or HRP-AF594-PLQD into 180 μL of substrate solution in a 96-well plate and monitored using a fluorescent plate reader. For GOX, 10 μL of 5 μg/mL HRP was added into the 180 μL substrate solution, and the reaction was subsequently initiated by adding 10 μL of 2 μg/mL GOX-AF594 or GOX-AF594-PLQD. For the colocalized enzymes, 10 μL of the prepared colocalized sample was added to 190 μL of substrate solution containing glucose and Amplex red to initiate the reaction. To compare with the free enzyme mixture, equivalent concentrations of each individual enzyme was added into the same substrate solution and used as a control.

9. Statistical Analysis. The mean and standard deviation data presented herein were the results of independent experiments that were performed in triplicate. Significant differences between groups were evaluated by a Student’s t test with p ≤ 0.05.

RESULTS AND DISCUSSION

1. Adsorption of Single Enzymes on PLQDs. The PLQD micelles were produced in a four-channel microreactor based on flash nanoprecipitation, in which the hydrophobic organic QDs were incorporated into self-assembled amphiphilic Pluronic triblock micelles. Representative TEM images of the QDs before and after incorporation into the Pluronic micelles are shown in Figure 2. The average size of the individual QDs is approximately 4–5 nm, and as shown, they were successfully incorporated within the micelles to form PLQDs.

It has been shown that amphiphilic polymer micelles have the capability of adsorbing proteins.31,32 For colocalization, it is known that the spatial arrangement and orientation plays a significant role in enabling efficient catalytic mechanisms.33,34 In general, smaller particles exhibit higher enzyme loading capacity because of their large surface area per unit volume. However, nanoparticles that are too small may significantly reduce the probability of colocalization of multiple enzymes on the same nanoparticles. The size of the individual QD nanoparticles and that of the Pluronic micelles ranged from 4 to 10 nm.35 It has been reported that on average, the loading capacity of one QD particle is up to 3 molecules of HRP.36 The DLS average size of the fabricated PLQD micelles was approximately 145 nm, which resulted in larger loading capacity for enzymes when compared to QD particles or Pluronic micelles. In the subsequent enzyme adsorption studies, the inner core of the PLQDs, which consists of the QDs and the hydrophobic segment of the polymer primarily attract the enzyme molecules and retain them on the micelles. The outer brushes, formed by the hydrophilic blocks of the polymer, also interact with the protein to prevent unfavorable conformational changes, leading to preservation of the enzymatic activity. The size of the micelles before and after adsorption of dye-conjugated enzymes was measured using DLS. As shown in Figure 3, while the adsorption of the dye-conjugated enzymes did not significantly affect the micelle size distribution, it increased the mean size of the micelles (Table 1). Overall, the micelle size increased with increasing enzyme concentration. The larger micelle size at high enzyme concentration may be attributed to a combination of compact loading and adsorption of multiple layers of enzymes onto the micelles. The free enzymes were not detectable in all adsorbed enzyme-dye conjugates samples, indicating the presence of free enzymes is negligible. In addition, when the enzyme concentrations were further increased (i.e., to twice the highest concentrations used), the size of the PLQD-enzyme micelles decreased to approximately 40–50 nm (data not shown). This apparent decrease may be attributed to oversaturation of the enzyme, and the smaller size is a result of a bimodal distribution of free dye-conjugated enzyme molecules and the PLQD-enzyme micelles. Therefore, the subsequent single enzyme kinetics studies were carried out at or below the higher enzyme concentration(s) to minimize the presence of free enzymes in solution.

2. FRET Study between QDs and Dye-Conjugated Enzymes. To further characterize single enzyme adsorption onto the micelles, FRET was studied to demonstrate adsorption of dye-conjugated enzymes on PLQD micelles. Typically, FRET occurs when the distance between the donor and the acceptor is within 10 nm.37 An excitation wavelength of 440 nm was used to maximize the excitation of QDs and
minimize the direct excitation of the dye. The fluorescence intensities of enzymes or dye-enzymes were normalized with the maximal value intensities of the PLQD micelles. The data in Figure 4 demonstrates that FRET occurred between the QDs and the AF594 dyes, as indicated by the quenching of the primary QD peak at 570 nm as well as the appearance of the sensitized dye peak at 615 nm by adsorption of the dye-conjugated enzymes. The quenching of the QDs and the sensitized dye fluorescence intensities increased with the enzyme-dye conjugate concentration. Figure S1 in the Supporting Information shows the comparison of the fluorescence intensities of the enzyme-dye conjugates before and after incorporation into the micelles. The normalized intensities of enzyme-dye conjugates adsorbed on PLQD micelles were obtained by subtracting the intensities of the PLQD micelles alone (i.e., no enzyme) to further demonstrate FRET between the QDs and AF594 dye-conjugated enzyme. The arrows indicate quenching of the primary peak at 570 nm and corresponding excitation of the AF594 dye. In control experiments, the QD peak was not quenched by adsorption of enzymes without dye labeling (red curve in Figure 4). From these experiments, it is reasonable to surmise that the presence of the dye caused significant quenching of the QD peak, which suggests that the distance between the dye and QD is 10 nm or less.

3. Catalytic Performance of Single Enzymes Adsorbed on PLQDs. Before performing colocalization studies, the catalytic performance of single enzymes adsorbed onto PLQDs was evaluated to ensure that enzyme activity was not affected by adsorption. Previous studies have shown that covalently immobilized GOX on magnetite nanoparticles has similar activity as free enzyme in solution, likely because conformational changes did not block access to the active site. To maximize the loading as well as enzymatic activity, the reaction was carried out overnight at 4 °C. In these experiments, the single enzymes were labeled with AF594 dye for consistency.

Figure 3. Size distributions of free enzyme dye conjugates and PLQD micelles characterized by DLS: (a) after adsorption of GOX-AF594 at three different concentrations and (b) after adsorption of HRP-AF594 at two different concentrations.

Table 1. Intensity-Averaged Size and Size Distributions of PLQDs and Enzyme-PLQDs

<table>
<thead>
<tr>
<th></th>
<th>0.1 μM GOX-AF594-PLQD</th>
<th>0.25 μM GOX-AF594-PLQD</th>
<th>0.625 μM GOX-AF594-PLQD</th>
<th>0.91 μM GOX-AF594-PLQD</th>
<th>2.27 μM HRP-AF594-PLQD</th>
</tr>
</thead>
<tbody>
<tr>
<td>size (nm)</td>
<td>143.0</td>
<td>144.0</td>
<td>147.2</td>
<td>156.1</td>
<td>148.6</td>
</tr>
<tr>
<td>standard deviation</td>
<td>28.6</td>
<td>2.6</td>
<td>1.4</td>
<td>0.7</td>
<td>6.7</td>
</tr>
</tbody>
</table>

a n = 3. Represents a statistically significant difference when compared with PLQD micelles (p ≤ 0.05).
The kinetics of the reaction catalyzed by each enzyme was evaluated to compare enzymatic performance of adsorbed enzyme vs free enzyme in solution. The amount of final product concentration in solution as a function of time was measured at each glucose substrate concentration using UV−vis spectroscopy. All of the initial reaction rates were estimated from the derivatives of a polynomial equation, which modeled the change in the resorufin concentration with reaction time. The data in Figure 5a were obtained by plotting initial reaction rates vs substrate concentration and indicate that the adsorbed and free enzymes displayed similar kinetic behavior. To determine the kinetic parameters, a Michaelis–Menten model was used and the \( K_m \) and \( v_{\text{max}} \) values of free and adsorbed GOX were estimated using a linear least-squares method. These values are shown in Table 2. The \( K_m \) values for both adsorbed and free GOX were 8.9 mM, which indicated that the affinity of the enzyme active site to the substrate did not change after adsorption. Likewise, the \( v_{\text{max}} \) values of both adsorbed and free enzyme were 11.1 μM/min. Overall, the GOX activity was retained after adsorption. Similar analyses were performed to obtain the kinetic parameters of free and adsorbed HRP (Figure 5b). As shown in Table 2, the \( K_m \) and \( v_{\text{max}} \) values for both free and adsorbed HRP were statistically indistinguishable, indicating that the adsorbed HRP also retained its activity.

### 4. Colocalization of GOX-AF594 and HRP-AF647 on PLQD Micelles.

To maximize the production of resorufin, the ratio of the concentrations of free GOX and HRP was varied from a GOX:HRP molar ratio of 1:4 to 1:40. The results indicated that resorufin production increased with increasing HRP concentration and a plateau was reached at a GOX:HRP ratio of 1:11 (data not shown). Based on these results, a GOX to HRP feed molar ratio of 1:11 was selected to perform the colocalization studies.

In these studies, an excitation wavelength of 594 nm was chosen to maximize the emission of QDs and to minimize the direct excitation of the AF 647 dye(s). When both HRP-AF647 and GOX-AF594 were mixed with the PLQD micelles, the primary AF594 peak at 620 nm was quenched (Figure 6) and the AF647 dye was excited with a peak at 670 nm in contrast to the situation when an equivalent concentration of GOX-AF594 was adsorbed onto the PLQD micelles. The normalized fluorescence intensities of samples with enzymes or dye-enzymes were normalized with the maximum intensity value of the 0.25 μM GOX-AF594-PLQD sample.

### Table 2. Comparison of Kinetic Parameters of Free Enzymes and Enzymes Adsorbed onto PLQD Micelles*

<table>
<thead>
<tr>
<th></th>
<th>GOX-AF594</th>
<th>GOX-AF594</th>
<th>HRP-AF594</th>
<th>HRP-AF594</th>
</tr>
</thead>
<tbody>
<tr>
<td>( v_{\text{max}} ) (μM/min)</td>
<td>11.1 ± 1.1</td>
<td>11.1 ± 0.8</td>
<td>9.0 ± 0.6</td>
<td>8.9 ± 1.1</td>
</tr>
<tr>
<td>( K_m ) (mM for GOX, μM for HRP)</td>
<td>8.9 ± 2.6</td>
<td>8.9 ± 1.1</td>
<td>21.8 ± 5.6</td>
<td>19.8 ± 5.6</td>
</tr>
</tbody>
</table>

*No statistically significant differences were observed between the parameters of the free enzymes and that of the adsorbed enzymes.
The enzymatic performance of the optimized colocalized GOX and HRP system was evaluated by measuring the kinetics of the coupled reaction catalyzed by this “artificial” multi-enzyme complex. Equivalent concentrations of free enzymes in solution were studied under the same experimental conditions as a control. The results shown in Figure 7 indicate that colocalizing the two enzymes on the same PLQD micelles enhanced the overall product conversion by 100%. Control experiments with micelles alone (i.e., no enzyme) ruled out any catalytic activity by the micelles themselves.

These results demonstrate the clear benefits of sequentially colocalizing multiple enzymes on nanoscale platforms. In many real-world systems, it is critical to colocalize enzymes so that reaction intermediates can rapidly find the next active site for the reaction to proceed. In situations where the intermediate product has a short lifetime, such a strategy may pay even more dividends. It is important to colocalize the enzymes within a few nm of each other (as demonstrated by FRET in this study) to enable production of the desired product. The biomimetic strategy outlined herein shows the value of using nanoscale platforms to accomplish this goal. This strategy is broadly applicable to other sequentially coupled multi-enzyme reactions by appropriately tailoring the nanoscale platform and colocalization methodology.

**CONCLUSIONS**

In this study, novel QD-embedded Pluronic-based micelles were designed to colocalize multiple enzymes. It was shown that adsorption of single enzymes led to FRET between the dye-labeled enzyme and the QDs. The catalytic activity of single enzymes was retained after immobilization. The occurrence of FRET between the two distinct fluorescent dyes that were used to label the two enzymes, when conjugated to the micelles, demonstrated that the enzymes were within a few nm of each other, which is indirectly indicative of colocalization on the same PLQD micelle. The colocalization of both enzymes on PLQD micelles enhanced the overall conversion rate by approximately 100% compared to the equivalent concentration of free enzymes in solution. This study describes the design of a nanoscale biomimetic materials platform for multi-enzyme colocalization and an effective strategy to characterize multi-enzyme immobilization and colocalization.

**ASSOCIATED CONTENT**

**Supporting Information**

Normalized intensities of enzyme dye conjugates adsorbed on PLQD micelles adjusted by subtracting the intensities of the PLQD micelles alone to further demonstrate FRET between the QDs and AF594 dye-conjugated enzyme: (a) GOX-AF594 and (b) HRP-AF647 (Figure S1). Normalized intensity of colocalized enzyme dye conjugates on PLQD micelles adjusted by subtracting the intensities of the GOX-AF594-PLQD micelles to further demonstrate FRET between the AF594 and AF647 (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

Notes

The authors declare no competing financial interest.

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**REFERENCES**


**ASSOCIATED CONTENT**

**Figure 7.** Resorufin conversion catalyzed by colocalized GOX-AF594 and HRP-AF647 on PLQD micelles compared to that catalyzed with equivalent concentrations of free GOX-AF594 and HRP-AF647 in solution, and with PLQD alone.