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Lyophilization of Semiconducting Polymer Dot Bioconjugates

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Abstract

Semiconducting polymer dot (Pdot) bioconjugates are a new class of ultrabright fluorescent probes. Here, we report a procedure for lyophilizing Pdot bioconjugates so that they successfully retain their optical properties, colloidal stability, and cell-targeting capability during storage. We found that when Pdot bioconjugates were lyophilized in the presence of 10% sucrose, the rehydrated Pdot bioconjugates did not show any signs of aggregation and exhibited the same hydrodynamic diameters as before lyophilization. The brightness of the lyophilized Pdots was at least as good as before lyophilization, but in some cases, the quantum yield of lyophilized Pdots curiously showed an improvement. Finally, by using flow cytometry, we demonstrated that lyophilized Pdot bioconjugates retained their biological targeting properties and were able to effectively label cells; in fact, cells labeled with lyophilized Pdot bioconjugates composed of PFBT, which were stored for 6 months at -80°C , were $\sim 22\%$ brighter than those labeled with identical but unlyophilized Pdot bioconjugates. These results indicate lyophilization may be a preferred approach for storing and shipping Pdot bioconjugates, which is an important practical consideration for ensuring Pdots are widely adopted in biomedical research.

INTRODUCTION

Fluorescence-based techniques are playing an increasingly important role in the study of biological systems. New fluorescent probes ranging from small organic fluorophores¹ to nanoparticles, such as quantum dots (Qdots),² and various forms of genetically encoded green fluorescent proteins (GFPs) have been developed.³ These fluorescent probes have made new measurements and advances possible but they have their limitations, such as low brightness, insufficient photostability, or toxicity concerns.^{4–6} As a result, there continues to be a need for probes that improve upon the existing fluorescent labels or at least complement them.

To meet this need, we and others have been developing Pdots as a new class of fluorescent nanoparticles.^{7–22} Compared to organic dyes and fluorescent proteins, Pdots possess orders of magnitude greater brightness and are more resistant to photobleaching.¹² When comparing to Qdots, for example, Pdots can be an order of magnitude brighter. Moreover, the dimensions of Pdots can be tuned from several to tens of nanometers without affecting

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SUPPORTING INFORMATION

SI contains: procedures of Pdots preparation, cell culture and immunofluorescence labeling, main chain structures of 6 semiconducting polymers, sizes and quantum yield values of lyophilized and unlyophilized Pdots. This material is available free of charge via the Internet at <http://pubs.acs.org>.

their spectral properties.¹³ Pdots with small sizes are desirable in situations where labeling with large nanoparticles may perturb the native behavior of the tagged biomolecules. The small Pdots may also be useful in crowded cellular or intercellular spaces where they can better penetrate and distribute themselves.²³ We have also developed various schemes to control the surface properties and bioconjugation of Pdots,^{9, 11, 12} which have enabled us to use Pdots for cell-surface and subcellular labeling,^{11, 12} as well as *in vivo* tagging of tumors in mice after tail-vein injection.¹⁰ In addition, we have developed various Pdot-based ratiometric sensors, including ones for pH, temperature.^{8, 22}

Although Pdots represent a promising new class of fluorescent probes, there are important practical considerations that will determine whether or not Pdots will be widely used. One consideration is the colloidal stability and shelf life of Pdots. Poor colloidal stability can be a significant drawback, and it has plagued other types of nanoparticles. Here, we report lyophilization, a freeze-drying / dehydration technique^{24, 25}, that can be used to prepare Pdot bioconjugates for long-term storage or shipping, provided the right conditions are used. Lyophilization is an important practical advance for making Pdots practical to use in biomedical research.

EXPERIMENTAL SECTION

Materials

Poly[(9,9-dioctylfluorenyl-2,7-diyl)-co-(1,4-benzo-(2,10,3)-thiadiazole)] (PFBT; MW 157000 Da; polydispersity, 3.0), Poly(9,9-dioctylfluorenyl-2,7-diyl) end capped with dimethyl phenyl (PFO, MW 120000 Da), poly[(9,9-dioctylfluorenyl-2,7-diyl)-co-(1,4-benzo-(2,1',3)-thiadiazole)] 10% benzothiadiazole (PF₁₀BT, MW 100000 Da) and poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-(1-cyanovinylene-1,4-phenylene)] (CNPPV, MW 15000 Da) were purchased from American Dye Source Inc (Quebec, Canada). PFBT directly functionalized with carboxylic acid (PFBT-COOH) groups and Boron dipyrromethene unit containing semiconducting polymer which emits at 690 nm (BODIPY 690) were synthesized in our lab.^{23, 26} Polystyrene-grafted ethylene oxide functionalized with carboxyl groups (PS-PEG-COOH) were purchased from Polymer Source Inc. (Quebec, Canada). Sucrose was ordered from Avantor Performance Materials (Phillipsburg, NJ, USA).

Preparation and characterization of Pdots

Pdots were prepared and conjugated with streptavidin using a method as reported earlier.¹² Detailed procedures can be found in SI. The hydrodynamic sizes of Pdots were measured with a dynamic light scattering (DLS) spectrometer (Malvern Zetasizer Nano ZS, Worcestershire, United Kingdom). Fluorescence quantum yields were collected using an integrating sphere (model C9920-02, Hamamatsu Photonics) with proper wavelength excitation. Fluorescence spectra of Pdots were taken with a Fluorolog-3 fluorospectrometer (HORIBA JobinYvon, NJ, USA).

Lyophilization

Solutions of Pdots were prepared at two different concentrations (4 and 20 nM) by diluting the Pdots solution with a buffer that was composed of 20 mM HEPES (pH 7.3), 0.1% (w/v) PEG and 0.05% (w/w) BSA. Sucrose was added to reach the desired final concentrations. The stock Pdots solutions were aliquoted into several vials (0.25 ml for 20 nM, 1.25 ml for 4 nM). Half of them were rapidly frozen in liquid nitrogen for 2 minutes, and were immediately placed under vacuum on a Labconco Freezone 6 freeze-dryer (Kansas City, MO, USA). After ~18 hr, lyophilized samples were removed from the freeze-dryer and were labeled as "lyophilized Pdots" and stored at -80 °C for a desired amount of time (from one day to 6 months). The other half of the aliquots were labeled "unlyophilized" and were

placed in a 4 °C refrigerator. For Pdots of each composition, the data was collected from the same batch.

Flow cytometry experiments

Measurements were performed on labeled cell samples prepared as previously described.¹² Cell culture and immunofluorescence labeling procedure can be found in SI. The flow cytometer BD FACSCanto II (BD Bioscience, San Jose, CA USA) was used. Cells flowing in the detection chamber were excited by a 488-nm laser. Side- and forward-scattered light were collected and filtered by a 488/10 nm band-pass filter, while fluorescence emission was collected and filtered by a 502-nm long-pass and a 530/30 nm (for PFBT and PFBT-COOH) or a 582/42 nm (for CNPPV) band-pass filter.

RESULTS AND DISCUSSION

For lyophilization, Pdots aliquots were first placed in the freeze-dryer. When the water component was completely removed, the Pdots were placed in -80 °C freezer for storage. After being stored for the desired amount of time (from 1 day to 6 months), a lyophilized aliquot was taken out of the freezer and the appropriate amount of water was added for re-dispersion. The final volume of the reconstituted solution was kept the same as that prior to lyophilization. For comparison, the unlyophilized aliquot stored at 4 °C was used. Both samples had the same composition and were measured with the same instrumentation. We tested various lyophilization conditions and chose the optimized procedure. We then made comparisons between the lyophilized and unlyophilized Pdot bioconjugates for hydrodynamic size, absorption spectra, emission spectra, quantum yield, and labeling efficiency. We also measured the hydrodynamic size, absorption spectra, emission spectra, quantum yield, and labeling efficiency of freshly prepared Pdots versus Pdots stored at 4 °C for one day (unlyophilized 1-day), and found them to be identical in all properties that we measured.

Size

We chose streptavidin conjugated PFBT Pdots (Strep-PFBT) to optimize the lyophilization recipe. The particular Strep-PFBT Pdots we prepared had a hydrodynamic diameter of 32 nm (Figure 1A), which was measured immediately after they were prepared. We then lyophilized the same Pdot-streptavidin conjugates without adding any reagents, and then re-hydrated the Pdots with water. Even after vigorous sonication, the hydrodynamic diameter of the rehydrated Pdots had increased to 220 nm, indicating that the lyophilization process had caused severe aggregation of the Pdots. Aggregated Pdot bioconjugates are not suitable for biological studies.

We modified our lyophilization recipe. First, we added 1% (w/v) sucrose. Here, we found the hydrodynamic diameter of the rehydrated Pdots was 50 nm (Figure 1C), which was much smaller than the 220 nm we had obtained without sucrose but it was still significantly larger than the original size of 32 nm. Sonication did not help further shift the size distribution of rehydrated Pdots to that before lyophilization. Therefore, the Pdot-streptavidin bioconjugates still were partially aggregated, albeit much less severely than without sucrose. We increased the concentration of sucrose to 10% (w/v). Figure 1D shows the size of the rehydrated Pdots returned to 32 nm. It should be noted that additional sonication was not needed after the rehydration procedure. The lyophilized Strep-PFBT Pdots used in the above measurements were stored at -80 °C for one day after lyophilization. We also tested lyophilized Strep-PFBT Pdots at two different concentrations (4 nM and 20 nM) that were stored for longer (1–6 months) at -80 °C. As shown in table S-1, the size remained at around 32 nm independent of concentrations after 6 months.

We also applied this lyophilization recipe to Pdots made of other semiconducting polymers (CNPPV, PFO, PF₁₀BT, BODIPY 690), including both streptavidin conjugated and unconjugated Pdots. The structures of the tested polymers are included in Figure S-1. We first measured the hydrodynamic sizes of unlyophilized Pdots stored over different durations (1–6 months). We then measured the hydrodynamic sizes of the different lyophilized Pdots stored for up to 6 months and compared them with that of their unlyophilized counterparts. As shown in table S-1, all the lyophilized Pdots possessed similar size to the unlyophilized Pdots after rehydration. To facilitate the bioconjugation reaction and dispersion in aqueous solution, the aforementioned Pdots were functionalized with carboxylic acid groups by doping PS-PEG-COOH during preparation. A new type of semiconducting polymers directly functionalized with low density carboxylic acid groups in the same polymer chain was also synthesized in our lab (PFBT-COOH, Figure S1).^{23, 26} With the directly incorporated carboxylic acid groups, PFBT-COOH Pdots offer a number of significant advantages, such as higher brightness and better colloidal stability.^{23, 26} The size of such prepared streptavidin conjugated PFBT-COOH Pdots after lyophilization was measured; we found the sizes of lyophilized Strep-PFBT-COOH Pdots stored for 1 month were the same as that of unlyophilized Pdots. The results in table S-1 show that lyophilized Pdots were easily re-dispersed back to their single particle form, independent of the concentration of the initial Pdot solution, even after being stored for up to 6 months. This indicates that the colloidal stability of streptavidin conjugated and unconjugated Pdots did not change after the lyophilization process with 10% sucrose. We also prepared and lyophilized Pdots dispersed in sucrose solution with concentration at 20% and 50% (w/v). We obtained similar results with 20% sucrose as we did with 10% sucrose, but we found 50% sucrose to be unsuitable for Pdot lyophilization. Instead of forming dry powders, Pdots lyophilized in the presence of 50% sucrose was left as sticky liquid with very high viscosity when it was taken out of the lyophilizer (Figure S-2). This observation suggests that the water component was not completely removed from the Pdot sample. Therefore, the lyophilization procedure described in the following sections was carried out with 10% sucrose. We note that simply freezing the Pdot solution and then thawing it when needed for use is not an appropriate approach for Pdot storage, because this process causes severe aggregation (Figure S-3).

Optical property

We measured the absorption and emission spectra of lyophilized Pdots after up to 6 months storage and compared them with that of their unlyophilized counterparts. As shown in Figure 2A–2C, 2F, the absorption and emission spectra of lyophilized streptavidin conjugated Pdots (Strep-PFBT, Strep-CNPPV, Strep-PFBT-COOH and Strep-BODIPY 690) were identical to their unlyophilized counterparts after being stored for up to 6 months. The same phenomenon was also observed in Pdots made of PFO (Figure 2D) and PF₁₀BT (Figure 2E). These results indicate lyophilization did not change the absorption and emission spectra of these conjugated and unconjugated Pdots.

We next studied the brightness of Pdots to determine if lyophilization had a negative effect on their fluorescence intensity. To facilitate the brightness comparison between lyophilized and unlyophilized Pdots of different concentrations, we measured their quantum yield (QY), because quantum yield is less dependent on concentration than fluorescence intensity. Table S-2 shows the quantum yield values of various Pdots that had and had not undergone lyophilization.

We found that the quantum yield of most lyophilized Pdots remained at the same level for the duration of the storage time. For example, the quantum yield of 20 nM lyophilized Strep-PFBT Pdots was 37% after stored for 1 day and 36% after 6 months storage, respectively. In contrast, the quantum yield of most unlyophilized Pdots showed a small but consistent decrease: when the same Pdots (20 nM Strep-PFBT Pdots) were stored unlyophilized, the

quantum yield decreased from 33% to 30% after 6 months storage. Similar quantum yield changes were also found in unconjugated PFBT and PFO Pdots. It is likely that oxidation of the semiconducting polymer, which would reduce the quantum yield, was minimized when the Pdots were lyophilized and stored at -80°C .

These results demonstrate that the brightness of Pdots certainly was not adversely affected by the lyophilization process, and remarkably, there could even be an enhancement in the optical performance of Pdots by going through the lyophilization procedure. For example, the quantum yield enhancement of 4 nM lyophilized Strep-PFBT Pdots over unlyophilized Strep-PFBT Pdots after 1 day storage was $(0.35-0.32)/0.32 = 9.4\%$. Although the mechanism that underlies this increase in quantum yield caused by lyophilization is unclear, we think the lyophilization process caused the internal rearrangement of the backbone of the semiconducting polymer.

Labeling efficiency

We tested the cell targeting capability of the Pdot bioconjugates to ensure they maintained their biological specificity. We used streptavidin-conjugated Pdots to label the cell surface receptor, EpCAM, which is an epithelial cell adhesion marker currently used for the detection of circulating tumor cells. We used flow cytometry to quantify the brightness of the cell labeling and the degree of non-specific absorption. For comparison between lyophilized and unlyophilized Pdot-streptavidin, we used 20 nM streptavidin conjugated Pdots (Strep-CNPPV, Strep-PFBT and Strep-PFBT-COOH) with 10% sucrose.

To ensure the sucrose did not have any unexpected effects on either cell labeling or non-specific absorption, we first carried out a control experiment where we quantified the cell labeling and non-specific absorption of Pdot-streptavidin in the presence and absence of 10% sucrose (see SI and Figure S-4). This control experiment showed that 10% sucrose did not have any noticeable effects on either positive cell labeling or non-specific absorption by Pdot-streptavidin.

We then compared the non-specific absorption and positive cell labeling by Pdot-streptavidin that had been lyophilized and stored over various time versus Pdots that were not lyophilized. Samples stored for 1 day after lyophilization were first tested. This experiment reports on any potential effect caused by undergoing the lyophilization process. As shown in the top panels in Figure 3, when cells were incubated with Pdot-streptavidin in the absence of the primary antibody (negative), the intensity peaks of both lyophilized (green curve) and unlyophilized (black curve) samples were low and comparable. This result confirmed that both lyophilized and unlyophilized Pdots produced very low amounts of non-specific binding in the absence of the primary antibody.

The data also show that the intensity peaks for cells labeled with both lyophilized (red curve) and unlyophilized (green curve) Pdots in the presence of primary antibody (positive) were well separated from that of the negative control samples. Specifically, for Strep-CNPPV and Strep-PFBT-COOH Pdots, the positive peak intensity values of lyophilized Pdots were similar to that of unlyophilized Pdots. For Strep-PFBT Pdots, the peak intensity value of lyophilized Pdots was a little larger than that of unlyophilized Pdots. The labeling brightness difference is consistent with our measured quantum yield values: for 20 nM Strep-CNPPV and Strep-PFBT-COOH, which were stored for 1 day, the QY values of lyophilized Pdots were similar to that of unlyophilized Pdots (table S-2); for Strep-PFBT, the cell labeling enhancement is 10%, which is similar to our measured quantum yield enhancement (9%).

We further tested the cell labeling efficiency of lyophilized and unlyophilized Pdots after long term storage. As displayed in the bottom panels in Figure 3, the intensity peaks for cells labeled with either lyophilized or unlyophilized Pdots stored for up to 6 months in the presence of primary antibody (positive) were still well separated from that of negative control samples. Specifically, for Strep-CNPPV Pdots, positive peaks of lyophilized and unlyophilized samples overlapped. For Strep-PFBT-COOH Pdots, positive peak of lyophilized sample was slightly higher than that of unlyophilized sample. For Strep-PFBT Pdots, the lyophilized sample showed a more noticeable brightness enhancement of 22%, which is consistent with our measured quantum yield enhancement of 20%. This result indicates that the lyophilization process did not impair the performance of Pdot bioconjugates, and for some semiconducting polymers, even enhanced their performance.

CONCLUSION

We studied the effect of lyophilization on the properties of Pdot-streptavidin bioconjugates, including colloidal stability, spectral properties, brightness, and labeling efficiency. Samples of various concentrations were stored for up to 6 months. We found that lyophilization with 10% sucrose was a good strategy to preserve Pdot bioconjugates. The rehydrated Pdots after lyophilization had the same size as that before lyophilization, even in the absence of sonication to help re-disperse the Pdots. The lyophilization procedure did not negatively affect the optical properties of Pdots. The quantum yield values of most lyophilized Pdots showed a consistent, albeit small, improvement in quantum yield after lyophilization; this phenomenon is likely caused by the rearrangement of the polymer backbone during lyophilization. The brightness of cells labeled with lyophilized Strep-PFBT Pdots stored for 6 months showed a 22% enhancement over the unlyophilized counterpart, likely because oxidation of the semiconducting polymer was minimized when the Pdots were lyophilized and stored at -80°C . We believe lyophilization will be a preferred route for the long-term storage of Pdots, which makes it an important practical consideration for the wide-spread adoption of bioconjugated Pdots in biomedical research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Dynamic light scattering measurements show the size distributions of streptavidin-conjugated PFBT Pdots. A) The size distribution of Pdots before lyophilization. B-D) The size distributions for rehydrated Pdots after lyophilization with (B) 0%, (C) 1%, and (D) 10% sucrose.

Figure 2.

Normalized absorption and fluorescence emission spectra of lyophilized and unlyophilized Pdots stored for up to 6 months. Pdots were made of PFBT (A), CNPPV (B), PFBT-COOH (C), PFO (D), PF₁₀BT (E) and BODIPY 690 (F). Pdots A, B, D and E were stored at -80°C for 6 months. Pdots C and F were stored at -80°C for 1 month. Solid curves are absorption. Dotted curves are fluorescence emission. The x-axis is wavelength with unit nm.

Figure 3.

Flow cytometry measurements of cells labeled with lyophilized and unlyophilized Pdots stored for up to 6 months. Column A: Strep-CNPPV; column B: Strep-PFBT; column C: Strep-PFBT-COOH. Red curve: Lyophilized positive; blue curve: Lyophilized negative; green curve: Unlyophilized positive; black curve: Unlyophilized negative. The x-axis is the relative fluorescence intensity. Pdots used in top figures were stored for 1 day. Pdots used in bottom figures were stored for a longer term: Strep-CNPPV 6 months; Strep-PFBT 6 months; Strep-PFBT-COOH 1 month. (1D: 1 day; 1 M: 1 month; 6 M: 6 months.)