Published in final edited form as:

Nanoscale. 2011 March ; 3(3): 1037–1045. doi:10.1039/c0nr00746c.

Incorporating Functionalized Polyethylene Glycol Lipids into Reprecipitated Conjugated Polymer Nanoparticles for Bioconjugation and Targeted Labeling of Cells

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Abstract

We report a simple and rapid method to prepare extremely bright, functionalized, stable, and biocompatible conjugated polymer nanoparticles incorporating functionalized polyethylene glycol (PEG) lipids by reprecipitation. These new nanoparticles retain the fundamental spectroscopic properties of conjugated polymer nanoparticles prepared without PEG lipid, but demonstrate greater hydrophilicity and quantum yield compared to unmodified conjugated polymer nanoparticles. The sizes of these hybrid nanoparticles, as determined by TEM, were 21–26 nm. Notably, these nanoparticles were prepared with several PEG lipid functional end groups and the biotin and carboxy moieties can be easily bioconjugated. We have demonstrated the availability of these end groups for functionalization using the interaction of biotin PEG lipid conjugated polymer nanoparticles with streptavidin. Biotinylated PEG lipid conjugated polymer nanoparticles bound streptavidin-linked magnetic beads, while carboxy and methoxy PEG lipid modified nanoparticles did not. Similarly, biotinylated PEG lipid conjugated polymer nanoparticles bound streptavidin-coated glass slides and could be visualized as diffraction-limited spots, while nanoparticles without PEG lipid or with non-biotin PEG lipid end groups were not bound. To demonstrate that nanoparticle functionalization could be used for targeted labeling of specific cellular proteins, biotinylated PEG lipid conjugated polymer nanoparticles were bound to biotinylated anti-CD16/32 antibodies on J774A.1 cell surface receptors, using streptavidin as a linker in a sandwich format. These data demonstrate the utility of these new nanoparticles for fluorescence based imaging and sensing.

Abstract

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We report a simple and rapid reprecipitation method to prepare extremely bright, functionalized, and biocompatible conjugated polymer nanoparticles (CPNs) incorporating functionalized polyethylene glycol (PEG) lipids. These 21–25 nm CPNs demonstrate greater hydrophilicity and quantum yield than unmodified CPNs and are highly stable in solution. We demonstrate bioconjugation and targeted delivery to antibody stained cells using biotin-functionalized PEG lipid PFBT nanoparticles.

Keywords

Bionanotechnology; Conjugated Polymers; Functional Coatings; Photoluminescence

1. Introduction

The use of highly fluorescent nanoparticles as labels for cellular imaging and *in vitro* assays is an extremely promising approach to maximize sensitivity and minimize the limit of detection. Such nanoparticles include inorganic semiconductor quantum dots (QDs)^{1, 2}, dye-doped silica particles³, and, and commercially available dye-loaded latex spheres. These nanoparticles offer numerous advantages over traditional organic dyes, including bright fluorescence and improved photostability. As a consequence, great efforts have been invested in preparation of highly fluorescent nanoparticles and their use in a wide variety of applications^{4–6}, including biosensing, live cell imaging, and intracellular dynamics. However, use of existing nanoparticles is not without disadvantages. For example, limited dye loading due to self quenching and undesirable leakage of small dye molecules has been reported for dye-doped silica nanoparticles³ and cytotoxicity due to leached metal from the nanocrystal core is a critical problem for use of QDs^{7–9}. While heavy metal leaching has been reduced by coating QDs with a variety of materials, such coatings can have their own associated cytotoxic effects^{7, 10} and may not completely ameliorate heavy metal leakage.

The limitations of current fluorescent nanoparticles provide impetus for the design of new nanoparticles with high photostability and bright fluorescence, but with greatly reduced cytotoxicity. One promising strategy is the development of conjugated polymer nanoparticles (CPNs). These nanoparticles are formed by collapse of highly fluorescent conjugated hydrophobic polymers with well known photophysical properties to form nanoparticles with high absorption cross sections and high radiative rates^{11, 12}. The result is extraordinarily bright fluorescent nanoparticles. Because these CPNs are composed of relatively benign constituents, they have low cytotoxicity¹³. Because their constituent conjugated polymers have intrinsic fluorescence, they cannot leach dye or constituent

materials. As a result, CPNs have established themselves as a useful optical probe for sensitive detection. Our laboratory is currently characterizing CPNs as markers of fluid phase uptake for cellular imaging and flow cytometry. However, the extreme hydrophobicity of CPNs leads to aggregation at high concentrations, thus limiting the amount of CPNs that can be added to cells in culture.

One approach to reduce the hydrophobicity of CPNs would be to introduce hydrophilic functional group(s) to the conjugated polymer starting material(s). However, this approach could alter the structure of the polymer and affect both optical properties and CPNs formation. Another strategy is to envelope the CPNs with hydrophilic component(s), without changing the structure of the polymer thus maintaining the optical properties of the polymer^{14, 15}. We were intrigued by reports that polyethylene glycol (PEG) with an attached phospholipid (PEG lipid) has been used to provide hydrophilicity to an otherwise hydrophobic nanosensor¹⁶, to polymer coated quantum dots^{17–20} and to semiconductor polymer nanospheres formed by miniemulsion^{21, 22}. We speculated that a similar strategy could be used with CPNs formed by reprecipitation. As PEG lipids are commercially available and PEG has been widely used in biological systems, surface modification of CPNs with functionalized PEG lipids is a viable method to create more hydrophilic nanoparticles. Importantly, PEG lipids can be functionalized with a variety of end groups to incorporate a moiety for linking biomolecular recognition elements to the CPN surface. As a result, functionalized PEG lipids not only improve the hydrophilicity and biocompatibility of CPNs for live cell imaging, but also allow specific labeling of cellular targets.

Here we report a general method that uses commercially available materials to prepare highly fluorescent CPNs that incorporate functionalized PEG lipids, using the straightforward reprecipitation method. The result is functionalized soluble nanoparticles of small size that are highly stable in aqueous solution over a large concentration range. The extremely bright fluorescence of these nanoparticles, coupled with functionality for targeted cellular imaging, gives them enormous potential for fluorescence based imaging and sensing, including applications with single nanoparticle detection limits.

2. Results and Discussion

2.1. Preparation of PEG lipid-modified conjugated polymer nanoparticles

CPNs form in response to rapid dilution of conjugated polymer solutions into water. The hydrophobic polymer molecules collapse in aqueous solution to create nanoparticles with very high intrinsic fluorescence. To prepare CPNs which incorporate PEG lipid into the nanoparticle structure, conjugated polymer solutions in THF were diluted into aqueous solutions containing functionalized PEG lipid, during brief mild sonication to aid mixing, as described in detail in the Experimental section. The PEG lipid molecules used contain two C₁₄ lipid chains linked to the PEG through the phosphate moiety to provide a bidentate hydrophobic group for interaction with conjugated polymer; the functional endgroup is located at the opposite end of the PEG chain.

PEG lipid-CPNs were prepared using PFBT and a series of PEG lipids (PEG M_r = 2000, 1000, 550) with either carboxy, biotin, or methoxy end groups (Table 1). Our intent was to

demonstrate that functionalized PEG lipid-CPNs can be prepared with a range of PEG sizes and moieties for bioconjugation using a common strategy. Size of PFBT nanoparticles formed under these conditions was characterized by TEM. Representative TEM data and size distribution are shown in Figure 1. Additional TEM data and size distributions are shown in Figure S1 (see Supporting Information). Diameters obtained from the TEM data for the different PEG lipid-PFBT nanoparticles are listed in Table 1. Mean PEG lipid-PFBT particle size is *ca.* 24 nm, and is insensitive to changes in the PEG lipid end groups and PEG M_r tested. We have also prepared PEG lipid-CPNs using other conjugated polymers, including PFO, PFPV, and MEH-PPV. These particles behave similarly to PEG lipid-PFBT nanoparticles, with variations in size (measured using DLS) and spectral properties that most likely reflect the differences between their respective conjugated polymer starting materials (Table S1; Supporting Information).

Size measurements determined by DLS, as reported here, reflect the hydrodynamic diameter, and are therefore expected to be higher than those measured by TEM, particularly for nanoparticles incorporating PEG lipids, since extension of long PEG groups into solution is expected to be accompanied by significant solvation not present under TEM conditions. However, absolute size values obtained by DLS measurements are accurate only for monodisperse particles, and measured sizes can be inflated by the presence of even small amounts of aggregate. For this reason, we use DLS size measurements here as a tool for comparison of relative size and do not interpret DLS data as absolute size values.

We also measured the zeta potential of the nanoparticles. The zeta potential of PEG lipid CPNs reflects the charge of the conjugated polymer, phospholipid, and any charge present on the endgroup. PFBT nanoparticles prepared with carboxy PEG lipid have negative zeta potential (-38 ± 1 mV), reflecting the the negative charge on both the phospholipid and charged endgroup. Biotin and methoxy PEG-lipid-CPNs have smaller negative zeta potentials (-9 ± 1 mV and -6 ± 1 mV, respectively), reflecting the neutral endgroup and negative charge on the phospholipid. These differences between CPNs prepared with different PEG lipid molecules provide initial evidence for PEG lipid incorporation into these CPNs.

Additional evidence for incorporation of PEG lipid into these CPNs comes from their observed properties. CPNs prepared with PEG lipid can be filtered through hydrophobic membrane filters in buffer (*e.g.* 0.2 micron PVDF syringe filters) without difficulty. Absorbance measurements of PEG lipid-CPNs before and after filtration are indistinguishable, indicating no measurable binding to the hydrophobic filter. In contrast, in our hands, CPNs prepared without PEG lipid and diluted in buffer bind to the filter in small but visible quantities, either as a result of their higher hydrophobicity, the possible presence of small aggregates, or possible instability in the presence of buffer salts. In addition, PEG lipid-CPNs will pass through a size exclusion column in buffer (*e.g.* 30 cm G-25 Sephadex packed column, commonly used in separations for bioconjugation methods) with high recovery, while unmodified particles show strong nonspecific binding to the stationary phase. Finally, we observe that PEG lipid-PFBT CPNs have somewhat higher quantum yield than than the corresponding unmodified CPNs (Table 2). For example, methoxy 550 M_r PEG lipid-PFBT nanoparticles have a quantum yield of $19 \pm 1\%$, compared to a measured

value of 12 ± 1 % for unmodified particles prepared using the same conjugated polymer and conditions; on average, PEG-lipid-PFBT nanoparticles have nearly a 50% increase in quantum yield relative to unmodified nanoparticles. In addition, Together, these observations are consistent with the incorporation of PEG lipid into the CPNs, with resulting increases in hydrophilicity and fluorescent brightness.

We hypothesize that PEG lipid-CPNs form via a process analogous to that for unmodified CPNs. According to the reprecipitation method, nanoparticles are formed when the hydrophobic polymer experiences a sudden change in the microenvironment of solvent, leading to collapse of the polymer chain into nanoparticles. We propose that in the presence of PEG lipid, the aliphatic side chains on the polymer backbone interact with the hydrophobic PEG lipid tail. When the polymer chain collapses, the PEG lipid is incorporated into the nanoparticle; the bidentate lipid tail is incorporated into the CPN core and is retained there by hydrophobic interactions, while the hydrophilic PEG group protrudes out into the aqueous environment. A similar structure has been proposed for polymer-encapsulated quantum dot nanoparticles coated with PEG lipid^{17, 18}. Hence, the CPN surface is modified with hydrophilic PEG polymer that help prevent aggregation, improve biocompatibility, and provide end groups that can be used for conjugation and labeling.

Our hypothesis of insertion of the lipid tail into the polymer chain during collapse is consistent with the observed higher quantum yield of the PEG lipid-CPNs. It is known that the fluorescence of conjugated polymers is quenched by interactions between polymer fluorophores²³. For example, polymer aggregation lowers the quantum yields of conjugated polymer in aqueous or hydrophilic solutions^{24–26}. Unmodified CPNs have lower quantum yields than their constituent conjugated polymer precursors, an effect which has been attributed to interactions between polymer segments after chain collapse²⁷. According to this analysis, the increase in quantum yield for PEG lipid-CPNs relative to unmodified CPNs can be rationalized on the basis of changes in the relative interactions of the polymer chain(s) in the CPNs caused by insertion of the lipid tail in the nanoparticle core. The lipid tails may create greater spacing between individual conjugated polymer fluorophores, leading to reduced intrachain quenching and correspondingly larger quantum yields than those observed for unmodified CPNs. In this case, the absorbance maxima of PEG lipid CPNs should also be decreased relative to unmodified particles, since decreased interaction of polymer fluorophores is accompanied by blue shifts in the absorbance spectrum²⁸. A comparison of high resolution absorbance spectra of methoxy 2000 M_r PEG lipid-CPNs and the corresponding particles prepared without PEG lipid demonstrates a decrease in absorbance maximum of 2.4 nm for PEG lipid CPNs (data not shown), consistent with disruption of interaction between conjugated polymer fluorophores by lipid insertion into the core. The presence of PEG lipid could also contribute to higher CPN quantum yield by exclusion of water from the nanoparticle surface.

We cannot rule out that PEG lipid-CPNs prepared here form by micelle entrapment of conjugated polymer nanoparticles, similar to those proposed for PEG-capped polymer coated Qdots²⁰ and semiconducting polymer nanospheres²¹. A study of micelle formation for an identical 2000 M_r PEG lipid without functional end groups reports a critical micellar

concentration (CMC) value that is approximately micromolar, with a measured micelle size of *ca.* 17 nm²⁹. Hence, at the PEG lipid concentrations used in our experiments (17–83 μ M), micelles may be present in solution prior to addition of conjugated polymer and mixing, although the thermodynamic stability of PEG lipid micelles of such small size is predicted to be low^{30, 31}, and our particles were not prepared under conditions that favor micelle formation. In control experiments designed to investigate the presence of lipid micelles in our nanoparticle preparations, no measureable light scattering was observed in PEG lipid solutions alone at concentrations up to 83 μ M (data not shown), indicating that micelles were not observable. It is unlikely that partitioning of independently precipitated CPNs into preformed PEG lipid micelles could occur, given that the previously reported PEG lipid micelle size is comparable to or smaller than the reported diameter for unmodified CPNs (*e.g.* 10 to 30 nm for PFBT¹²), although we cannot rule out this possibility. If CPNs are present inside larger than predicted micelles, there must be intimate association of the lipid tails with the CPN structure sufficient to produce the observed increased quantum yield. In this case, the final PEG lipid-CPN structure would be indistinguishable from that resulting from the proposed coprecipitation mechanism.

2.2. Optimization of PEG lipid-CPN Preparation

To determine preparation conditions that result in maximal incorporation of CPNs with PEG lipid, experiments were carried out where we increased the concentration of PEG lipid in solution. While we initially nanoparticle preparations used 50 μ g/ml PEG lipid in aqueous solution during nanoparticle preparation, PEG lipid-CPNs were also prepared using 25 μ g/ml, 100 μ g/ml, 150 μ g/ml, and 200 μ g/ml PEG lipid. No significant change in apparent hydrodynamic size of the resulting nanoparticles was observed by DLS relative to that for nanoparticles prepared in the original 50 μ g/ml PEG lipid concentration (Table 3). In contrast, when nanoparticles were prepared in reduced concentrations of PEG lipid (less than 20 μ g/ml), a portion of the nanoparticle preparation bound to the membrane filter, reflecting increased hydrophobicity that presumably results from limited incorporation of PEG lipid into CPNs. The size data suggest that maximum incorporation of the PEG lipids tested here is achieved for preparations that use a 50 μ g/ml PEG lipid solution.

To see the dependence of PEG lipid-CPN size on initial polymer concentration, PEG lipid-PFBT nanoparticle solutions were prepared from initial conjugated polymer concentrations of 10–250 ppm in THF via a ten-fold dilution to final concentrations ranging from 1 to 25 ppm (Table 4). The size of the resulting nanoparticles were evaluated by DLS. At these starting concentrations of conjugated polymer (10 to 250 ppm), the apparent hydrodynamic diameter of the PEG lipid-PFBT nanoparticles are independent of the starting concentration, and the only outcome of increased starting polymer concentration is increased nanoparticle concentration. However, at high concentrations, apparent particle size increases. Ten-fold dilutions of polymer concentrations above 500 ppm resulted in larger observed particle sizes by DLS. This size increase is a function of starting polymer concentration rather than final CPN concentration, since 100-fold dilutions of 1000 ppm polymer (to create final Np concentrations of 10 ppm) resulted in similar increased particle size (Table 4). It has been observed that the size of unmodified CPNs also varies with starting polymer concentration¹².

2.3. Fluorescence Properties of PEG lipid-CPNs

We evaluated the spectroscopic properties of PEG lipid-CPNs. Notably, absorption and emission maxima of PEG lipid-CPNs (Figure S2; Supporting information) are very close to those of particles prepared in the absence of PEG lipid for PFBT, PFO, PFPV, and MEH-PPV PEG lipid nanoparticles^{12, 27, 32}. Like unmodified CPNs the emission maxima of nanoparticles in aqueous solution are slightly red shifted compared to precursor conjugated polymer dissolved in organic solvent (THF), while the overall shape of the emission profile is maintained. This phenomenon has been attributed to a change in the spatial environment of the polymer fluorophores caused by folding to create the nanoparticle^{27, 32}. As noted above, PEG lipid-CPNs are somewhat less red-shifted than bare particles (by 2.4 nm), presumably as a result of lipid insertion into the folded core. Like unmodified CPNs, PEG lipid-CPNs show good photostability (Figure S3; Supporting Information); in particular, PEG lipid-PFBT nanoparticles show the best photostability in our experiments (Figure S3) which agrees with previous measurements of unmodified CPNs¹². Together, these data indicate that apart from increases in quantum yield, addition of PEG lipid does not substantially alter the spectroscopic behavior of CPNs.

2.4. Stability of PEG lipid-CPNs in solution

Over time or at high concentrations, hydrophobic particles will tend to form small aggregates as a mechanism for water exclusion from hydrophobic surfaces. Hence, minimization of aggregation is desirable to increase nanoparticle shelf life. Since incorporation of a PEG lipid into CPNs results in increased surface hydrophilicity, our expectation was that once formed, these PEG lipid-CPNs would be highly stable in solution. We have observed no signs of aggregation in any of the PEG lipid CPN solutions described here. However, to more thoroughly assess stability over time, the apparent diameter of CPNs in a 28 ppm solution of carboxy PEG lipid-PFBT nanoparticles was measured by DLS at intervals over 60 days (Figure 2). No significant variation in apparent hydrodynamic size was observed, indicating that observable aggregation did not occur, and that these PEG lipid-CPNs are stable for long periods of time in solution. The resistance of these PEG lipid-CPNs to aggregation may reflect the surface charge contributed by the PEG lipid and end groups, steric effects of PEG interactions, and the increased hydrophilicity of the PEG.

Our standard protocol (10-fold dilution of 250 ppm conjugated polymer into 50 ppm PEG lipid) yields 28 ppm CPNs of reproducible size after evaporation of the THF. However, higher concentrations could be advantageous for specific applications, including chromatographic separations during bioconjugation and live cell experiments requiring dilution of CPN stock into media. As a result, we investigated the stability of PEG lipid nanoparticles to concentration by ultrafiltration using a centrifugal concentrator. In these experiments, a 28 ppm solution of carboxy PEG lipid-PFBT nanoparticle solutions were concentrated to a final concentration of 625 ppm. Portions of this concentrated solution were rediluted to 25 ppm before analysis by DLS. The resulting apparent hydrodynamic size (60 ± 2 nm) was indistinguishable from the size of original solutions (59 ± 2), indicating that no aggregation occurred as a consequence of concentration. No binding of CPN solutions to the concentrator filters could be observed. While we have not concentrated PEG lipid-CPNs above 625 ppm, we expect that even higher concentrations of these particles are achievable.

In contrast, unmodified CPNs cannot be concentrated by ultrafiltration due to nonspecific binding to the available ultrafiltration membranes, and are currently concentrated by dilution in glycerol followed by vacuum evaporation³³ to yield CPN solutions of nanoparticles in glycerol instead of water or buffer, with an upper concentration limit of about 200 ppm.

2.5. Bioconjugation of PEG lipid-CPNs

Conjugation of nanoparticles to specific biomolecules such as antibodies or other biomarkers is highly desired for specific labeling of biomolecules on or within the cell. The PEG lipid end groups contain inherent functionality for molecular recognition and/or covalent linkage. To demonstrate that these end groups are in a steric and conformational arrangement that allows bioconjugation, we carried out a series of experiments in which streptavidin was used to bind PFBT PEG lipid-CPNs with biotin end groups. In the first set of experiments, biotin modified CPNs were incubated with magnetic streptavidin beads. After pulling out the beads from solution and washing to remove unbound nanoparticles, nanoparticles bound to the beads were removed by competition with free biotin and the magnetic beads removed with a strong permanent magnet. As shown in Figure 3, supernatant from the magnetic beads contains significant nanoparticle fluorescence (solid line), indicative of biotin-functionalized PEG lipid-CPNs binding to the streptavidin beads. In contrast, no nanoparticle fluorescence was observed in the supernatant from magnetic beads incubated with carboxy-PEG lipid-CPNs (Figure 3; dashed line). These results demonstrate both successful incorporation of biotin PEG lipid into PFBT CPNs, and the availability of the PEG lipid endgroup for molecular recognition and/or covalent linkage.

In additional experiments, a streptavidin coated cover glass was incubated with a very dilute solution of nanoparticles modified with biotin PEG lipid, and then rinsed to remove non-binding particles. As shown in Figure 4, significant numbers of near diffraction-limited spots of nanoparticle fluorescence can be observed (Figure 4A), indicating biotin-functionalized PEG lipid CPNs binding to streptavidin. In contrast, very little nanoparticle fluorescence is observed in the control plates incubated with PEG lipid-CPNs with carboxy end groups (Figure 4C). Together, these data indicate binding of biotin PEG lipid-CPNs to the streptavidin coated glass.

Surprisingly, careful examination of the CPN signal observed in these experiments suggests possible observation of single nanoparticles. The diffraction limit of our microscope is 225 nm. Hence, we cannot distinguish the signal from individual nanoparticles if they are less than 225 distance apart. In this case, variations in the intensity of individual sites of PFBT fluorescence can be used to indicate varying numbers of CPNs in the different diffraction-limited spots. We estimate that a signal from a single nanoparticle of diameter 25 – 50 nm (TEM vs. DLS diameter) could occupy one to four pixels in these images, depending on whether the nanoparticle were located in the center or periphery of individual pixels. We examined the intensity of all image objects occupying four or fewer pixels. As shown in Figure 4B, the intensity distribution of the near diffraction-limited regions show a narrow distribution of well separated biotin PEG lipid-CPN spots of approximately constant intensity (Figure 4B), consistent with measurement of single particles. We cannot distinguish between single particles and small aggregates of consistent size under these

conditions. However, given the lack of aggregation evident in the TEM data (Figure 1), the observed size stability of these nanoparticles in solution over time (Figure 2) and the precedent for nonaggregation of PEG coated particles in previously published systems^{19, 20}, formation of aggregates is not expected here. A substantive conclusion of single particle imaging under these conditions requires additional experimentation. However, the possibility of single particle imaging data obtained here with a standard camera and arc lamp excitation highlights the extreme brightness of these PEG lipid-CPNs and their potential utility for single particle imaging in biological systems.

2.6. Targeting of Bioconjugated PEG lipid CPN to CD16/32 Receptors

The properties of PEG lipid-CPNs such as their high extinction coefficient, bright fluorescence, photostability, and functionalization indicates significant potential for targeted single particle imaging and tracking in living cells. Here we demonstrate targeted localization of functionalized nanoparticles to individual CD16/32 receptors on the surface of mouse macrophage J774A.1 cells. In this case, a commercially available biotin-linked rat anti-CD16/32 antibody was bound to CD16/32 on the cell surface, and then labeled with biotin-functionalized PEG lipid PFBT nanoparticles, using streptavidin as a linker. The result was Ab-conjugated nanoparticles that specifically labeled the antibody tagged receptors on the cell surface. Figure 5 shows the differential interference contrast and fluorescence images taken of labeled cells. Localized nanoparticle fluorescence is observed on the periphery of the cell, typical for membrane localization, indicating binding of biotinylated nanoparticles to the cell surface via a streptavidin linker in a sandwich format. Control experiments performed either without streptavidin or using carboxy modified nanoparticles instead of biotinylated nanoparticles showed no fluorescence (data not shown). Together, these data demonstrate that PEG lipid-CPNs can target specific tagged proteins.

3. Conclusions

Incorporation of functionalized PEG lipid-CPNs is a simple method to prepare extremely bright biocompatible nanoparticles with enhanced properties suitable for fluorescence imaging applications. The PEG lipids impart improved hydrophilicity and quantum yield, and straightforward conjugation to biomolecules for targeted delivery. We have demonstrated the utility of bioconjugation via PEG lipid biotin end groups. The resulting data demonstrates that functional end groups on PEG lipid-CPNs provide a platform to conjugate nanoparticles to molecules of biological importance. Hence, PEG lipid-CPNs are a viable technology for a wide range of labeling and imaging applications in living biological systems.

4. Experimental

Reagents

The polyfluorene conjugated polymers PFBT (poly[(9,9-dioctylfluorenyl-2, 7-diyl)-*co*-(1,4-benzo-[2,1',3']-thiadiazole)], MW 48,000, polydispersity 2.7), PFPV (poly[(9,9-dioctyl-2,7-divinylene)fluorenylene]-*alt-co*-[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene]), MW 85,000, polydispersity 5.4), MEH-PPV (poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-

phenylenevinylene]-end capped with DMP, MW 565,000, polydispersity 5.1) and PFO (poly[(9,9-dioctylfluorenyl-2,7-diyl)]-end capped with DMP, MW 29,000, polydispersity 3.0) were purchased from American Dye source, Inc (Quebec, Canada). M_r 2000 PEG lipids with biotin end groups (1,2-Dimyristoyl-sy-Glycerol-3-phosphoethanolamine-N-[biotinyl (polyethylene glycol)-2000];(Ammonium salt)), and carboxy end groups (1,2-Dimyristoyl-sy-Glycerol-3-phosphoethanolamine-N-[carboxy (polyethylene glycol)-2000];(ammonium salt) and 550, 1000, and 2000 M_r PEG lipid with methoxy end groups (1,2-Dimyristoyl-sy-Glycerol-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-550, 1000, or 2000] were purchased from Avanti Polar Lipids. THF (anhydrous HPLC grade) was obtained from Fisher Scientific. Biotin rat anti- mouse CD16/CD 32 antibody was purchased from BD Pharmingen. All chemicals and biological molecules were used without further purification.

Method for Preparation of Nanoparticles

PFO, PFPV, PFBT, and MEH-PPV CPNs were prepared with biotin, carboxy, amine, and methoxy functionalized PEG lipids (550, 1000, or 2000 MW PEG). The stock solutions of conjugated polymers (1000 ppm) were dissolved in HPLC grade THF by stirring overnight. Next, functionalized PEG lipids were dissolved in distilled-deionized H₂O (ddH₂O) at concentrations between 25–250 ppm. The conjugated polymer nanoparticles were then prepared by rapidly dispersing 1 ml of conjugated polymer solution (10–250 ppm) into 9 ml of the PEG lipid solution under continuous mild sonication (45% amplitude) with a microtip-equipped probe sonicator (Branson, 4C15) for two minutes. THF was evaporated under vacuum overnight at room temperature. Finally, the nanoparticles were filtered through a 0.2 µm PVDF syringe filter.

Nanoparticle Characterization

The nanoparticles were evaluated by transmission electron microscopy (TEM) and dynamic light scattering. A Hitachi H7600T TEM at 120 kV and a cryostage at liquid nitrogen temperatures was used for all TEM measurements. Samples were prepared by drop casting nanoparticle solutions onto formvar/carbon grids. CPN diameter was measured with Image J. The measured particle diameters were fit to a Gaussian distribution using SigmaPlot (Systat).

Dynamic light scattering was performed using a Malvern Zetasizer (ZS90) at 25°C using distilled-deionized H₂O (ddH₂O) as dispersant. Prior to each DLS measurement, samples were briefly sonicated in a bath sonicator for 30 seconds to remove bubbles and minimize aggregates. The Z-average and polydispersity index were determined using cumulants analysis and manufacturer supplied software. Data were analyzed in terms of intensity weighted distributions. Three runs were performed for each sample, and the mean and standard deviation of both the Z-average and polydispersity index were calculated. Fluorescence emission spectra of the CPNs were acquired using a photon counting spectrofluorometer (Photon Technology International; QM-4). The fluorescence emission of PFBT nanoparticles was measured from 480 nm – 700nm in aqueous solution using 460 nm excitation. PFO nanoparticles were excited at 384 nm and emission measured from 395 – 700nm. PFPV nanoparticles were excited at 458 nm and emission measured from 480nm – 700nm. MEH-PPV nanoparticles were excited at 498 nm and emission measured from 510 –

725nm. Both excitation and emission monochromator slits were set to achieve a 4 nm band pass. Absorbance spectra were recorded using a Genesys 10UV Scanning spectrophotometer (Thermo) using a 1 cm quartz cuvette. Individual quantum yields were calculated using fluorescein in 0.1 M NaOH as a standard. High resolution spectra of methoxy 2000 M_r PEG lipid-PFBT and bare PFBT particles were acquired using a UV-2501PC (Shimadzu) scanning spectrophotometer with 0.5 nm spectral resolution.

Nanoparticle concentrations were estimated from the mass of conjugated polymer starting material diluted into aqueous solution, assuming complete polymer to nanoparticle conversion. Specifically, nanoparticle volumes were calculated from the particle diameter measured by TEM, assuming a spherical shape. Nanoparticle mass was converted to nanoparticle mass assuming a nanoparticle density of 1 g/cm³ (actual density is between 0.95 and 1.05 g/cm³). Dividing the total mass of conjugated polymer used in the reprecipitation by the mass of a single nanoparticle then yielded the number of nanoparticles formed, which was easily converted to moles of nanoparticles and molar concentration of the nanoparticle suspension. . Concentration calculations do not take into account small reductions in yield that result from filtration and may therefore be a slight overestimate. UV measurements taken before and after filtration indicate that loss from filtration is small (a few percent).

Concentrating PEG lipid modified nanoparticles

Solutions containing dilute PEG lipid-CPNs were concentrated by ultrafiltration using a 30 kD cutoff centrifugal concentrator with a regenerated cellulose filter (Millipore) according to manufacturer protocols. Solutions were concentrated up to 625 ppm.

Streptavidin Pull-down of Biotin PEG Lipid-PFBT nanoparticles

PFBT nanoparticles (28 ppm) prepared with biotin functionalized PEG lipid were incubated with magnetic streptavidin beads (New England Biolabs) for 30 mins in phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄). The magnetic beads were pulled down using a strong permanent magnet and washed 5X in PBS to remove possible unbound CPNs. The magnetic beads were incubated overnight with 0.2 mg/ml biotin to competitively remove bound nanoparticles then removed using magnetic field. The fluorescence from the remaining biotinylated nanoparticles was measured using 460 nm excitation while scanning the fluorescence emission from 480 – 700 nm. The slits for both the excitation and emission monochromators were set to achieve a 2 nm band pass.

Imaging of biotinylated nanoparticles localized on streptavidin-coated cover glass

For single particle fluorescence imaging, cover glasses were cleaned with concentrated sulfuric acid, washed with water and dried in air. Clean cover glasses were then coated with 1% poly-L-lysine, washed with water to remove excess poly-L-lysine, incubated with 1 mg/mL streptavidin for 30 minutes, and carefully washed with Ringer's Buffer 3X (RB; 155 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM NaH₂PO₄, 10 mM glucose, 10 mM HEPES, pH 7.2). Dilute solutions (5 pM / 25 ppb) of CPNs (carboxy PEG lipid-PFBT and biotin PEG lipid-PFBT) were incubated with the streptavidin-modified cover glass by inverting the cover glass over a drop of dilute CPNs on parafilm. Cover glasses were

incubated with PEG lipid-CPNs for half an hour, washed carefully 3X with RB buffer, and air dried before taking images. Fluorescence imaging was performed by inverted epifluorescence microscope (Olympus IX71, 60X/1.45 N.A. objective using Xe arc lamp excitation, 495/10 nm excitation filter, and 510 nm long pass emission filter).

Targeting of PEG lipid-PFBT nanoparticles to cell surface receptors

J774A.1 macrophage cells were plated onto 35 mm glass bottom microscope dishes in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% fetal bovine serum, 1% penicillin-streptomycin and 1% glutamate and incubated in humidified environment overnight (5% CO₂, 37 °C). Adherent cells were washed with RB 3X, fixed with 4% paraformaldehyde for 10 minutes at 37 °C, and blocked with 1% bovine serum albumin (BSA) for 1 hour at room temperature, washed again, and treated with 1:1000 dilution of biotin rat anti- mouse CD16/CD32 antibodies (BD Pharmingen) for 2 hours. Cells were washed 3X with RB to remove unbound antibodies before incubating with 1 µg/ml streptavidin for 30 minutes, again at room temperature. After streptavidin incubation, cells were washed 3X with RB, then incubated with PEG lipid-CPNs (biotin 2000 M_r PEG lipid-PFBT or carboxy 2000 M_r PEG lipid-PFBT; 5 pM / 25 ppb) for 30 minutes, followed by an additional three washes with RB. Images were acquired with an inverted microscope [Olympus IX71, Xe arc lamp for excitation and filters and beam splitters (495/10 for excitation and 510 LP for emission) from Chroma, Ocra- ER CCD (Hamamatsu). The acquired images were analyzed using Slidebook 5.0

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Dr. George Chumanov for use of his high resolution spectrophotometer and Dr. Jason McNeill for helpful discussion of the manuscript. Financial support for this project came from the National Institutes of Health (1R01GM081040). Supporting Information is available online from Wiley InterScience or from the author.

Abbreviations

PFBT	poly[(9,9-dioctylfluorenyl-2, 7-diyl)- <i>co</i> -(1,4-benzo-{2,1',3}-thiadiazole
PFPV	poly[(9,9-dioctyl-2,7-divinylene fluorenylene)-alt-co-{2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene}]
MEH-PPV	poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene vinylene]
PFO	poly[(9,9-dioctylfluorenyl-2,7-diyl)]
CPN	conjugated polymer nanoparticle
PEG	polyethylene glycol
DLS	Dynamic Light Scattering

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Figure 1. Representative TEM images and size distribution for PEG lipid-PFBT nanoparticles (A) TEM image of methoxy 2000 M_r PEG lipid-PFBT nanoparticles. Images were acquired at 120 kV on a cryostage at liquid nitrogen temperature and are shown at 120,000 magnification. Scale bar is 500 nm. Some blurring of the borders between these small nanoparticles is an artefact of high magnification. (B) Histogram of size distribution and fit to a Gaussian function for the methoxy 2000 M_r PEG lipid-PFBT nanoparticles showing the average diameter to be 24 ± 5 nm (mean $\pm$ FWHM).

Figure 2. Apparent Hydrodynamic size of biotin PEG lipid-PFBT nanoparticles as a function of time

Hydrodynamic size was monitored by DLS over 60 days. All measurements were in triplicate. Error bars are the standard deviation and are inclosed within the data symbol. Note that x-axis is not linear.

Figure 3. Fluorescence emission spectrum of biotinylated PEG lipid PFBT nanoparticle pulldown with streptavidin magnetic beads

Streptavidin coated magnetic beads were incubated with biotinylated 2000 M_r PEG lipid PFBT nanoparticles, washed to remove unbound CPNs, and then incubated with free biotin to release bound nanoparticles. Magnetic beads were removed with a strong magnet, and the fluorescence of the supernatant was recorded (solid line; $\lambda_{\text{ex}} = 460 \text{ nm}$). Control experiments using carboxy 2000 M_r PEG lipid-PFBT nanoparticles do not bind streptavidin magnetic beads, as shown by the emission spectrum of the control supernatant (dashed line).

Figure 4. Single nanoparticle fluorescence and intensity distributions from biotinylated PEG lipid-PFBT nanoparticles bound to streptavidin coated cover glass

(A) Representative fluorescence image of streptavidin-coated cover glass incubated with biotinylated 2000 M_r PEG-lipid-PFBT nanoparticles ($\lambda_{\text{ex}} = 495 \text{ nm}$; $\lambda_{\text{em}} = 510 \text{ nm}$ long pass filter). Scale bar = 10 μm . (B) histogram of biotinylated 2000 M_r PEG-lipid-PFBT nanoparticle fluorescence intensities obtained from the image; a threshold mask was applied to all objects (C) fluorescence image of streptavidin-coated glass slide incubated with carboxy 2000 M_r PEG lipid-PFBT nanoparticles, as a control for nonspecific binding to slide and (D) histogram of the 2000 M_r PEG lipid-PFBT nanoparticle fluorescence

intensities obtained from the image. Exposure times were identical for (A) and (C). The diffraction limit of the microscope was 225 nm.

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Figure 5. Biotinylated PEG lipid-PFBT nanoparticles targeted to cell surface receptors
(A) Differential Interference Image (DIC) of fixed J774A.1 cells; (B) fluorescence image of fixed J774A.1 cells labeled with biotinylated PFBT nanoparticles. Scale bar is 50 μ m. J774 A1 macrophage cells which express CD16/32 (F_c receptor) were paraformaldehyde fixed and incubated with biotinylated anti-CD16/32 antibody. After washing the cell with RB, the Cells were next incubated with streptavidin, washed, and labeled with biotinylated PEG lipid-PFBT nanoparticles. Images were obtained with 495 nm excitation, using a 510 nm long pass emission filter.

Table 1

Size of PFBT nanoparticles prepared with different varieties of PEG lipids by TEM.

Nanoparticle	Diameter (mean $\pm$ FWHM; nm)
Methoxy 550 M _r PEG lipid-PFBT	26 $\pm$ 5
Methoxy 1000 M _r PEG lipid-PFBT	26 $\pm$ 4
Methoxy 2000 M _r PEG lipid-PFBT	24 $\pm$ 5
Biotin 2000 M _r PEG lipid-PFBT	21 $\pm$ 5
Carboxy 2000 M _r PEG lipid-PFBT	24 $\pm$ 5

Table 2

Quantum yields for PFBT CPNs (fluorescein in 0.1 M NaOH as reference).

Nanoparticle	Quantum Yield
Methoxy 550 M _r PEG lipid-PFBT	19±1%
Biotin 2000 M _r PEG lipid-PFBT	17±1%
Carboxy 2000 M _r PEG lipid-PFBT	18±1%
PFBT (unmodified)	12±1%

Table 3

Apparent hydrodynamic size for carboxy 2000 M_r PEG lipid-PFBT CPNs prepared with a range of starting PEG lipid concentrations

PEG lipid	Diameter (DLS; nm)	Polydispersity Index (DLS)
25 µg/ml	52	0.25
50 µg/ml	54	0.22
100 µg/ml	52	0.23
150 µg/ml	58	0.22
200 µg/ml	56	0.19

Table 4

Apparent hydrodynamic size for carboxy 2000 M_r PEG lipid-PFBT CPNs prepared from a range of starting conjugated polymer concentrations.

[PFBT] _i	[PFBT] _f	Dilution factor	Diameter (DLS; nm)	Polydispersity Index (DLS)
1000 ppm	1 ppm	1000	86	0.35
500 ppm	50 ppm	10	62	0.15
250 ppm	25 ppm	10	59	0.15
200 ppm	20 ppm	10	59	0.17
150 ppm	15 ppm	10	58	0.19
100 ppm	10 ppm	10	58	0.19
50 ppm	5 ppm	10	58	0.19
10 ppm	1 ppm	10	58	0.22