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PASSIVATION AND FUNCTIONALIZATION OF CONJUGATED POLYMER NANOPARTICLES WITH HEAD GROUP MODIFIED PHOSPHOLIPIDS AND PROTEINS

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PASSIVATION AND FUNCTIONALIZATION OF CONJUGATED POLYMER NANOPARTICLES WITH HEAD GROUP MODIFIED PHOSPHOLIPIDS AND PROTEINS

A Dissertation
Presented to
The Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Chemistry

by
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August 2014

Accepted by:
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ABSTRACT

Conjugated polymer nanoparticles (CPNs) possess important characteristics such as high fluorescence brightness, reasonable photostability, and non-toxicity. These properties allow the use of CPNs in fluorescence based cellular and biological applications including cellular labeling, imaging, biosensing, and single particle tracking. To realize the broad applications of CPNs, it is required that CPNs possess functionality to conjugate a recognition moiety and maintain colloidal stability in biological media. In the following dissertation, we have prepared functionalized CPNs by surface passivation with head group-functionalized poly (ethylene glycol) lipids and proteins. We studied the colloidal stability of CPNs in biological media and investigated their utility as cellular labels, fluid phase markers and detection reagent in immunoassay.

Chapter 1 summarizes the general background information of CPNs including methods of preparation, physical properties, bioanalytical applications, and functionalization strategies.

Chapter 2 contains a systematic study of a simple and rapid method to prepare extremely bright, functionalized, stable and biocompatible conjugated polymer nanoparticles incorporating functionalized polyethylene glycol (PEG) lipids by reprecipitation. The size of these nanoparticles, as determined by TEM, was 24±5 nm. These nanoparticles retain the fundamental spectroscopic properties of conjugated polymer nanoparticles prepared without PEG lipids, but demonstrate greater hydrophilicity and quantum yield compared to unmodified conjugated polymer nanoparticles. Nanoparticles were prepared with several PEG lipid functional end groups,
including biotin and carboxy moieties that can then be conjugated to biomolecules. We have demonstrated the availability of these end groups for functionalization using the interaction of biotin PEG lipid conjugated polymer nanoparticles with streptavidin. 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-CD 16/32 antibodies on J774A.1 cell surface receptors, using streptavidin as a linker. This work represents a method of preparation of bright and biocompatible CPNs by inclusion of functionalized PEG lipids. The functional end group on PEG lipid CPNs offers a link to conjugate CPNs to biomolecules. Hence, PEG-lipid CPNs are a viable technology for targeted labeling and imaging in biological systems.

Chapter 3 constitutes the comparative study of sensitivity and limit of detection using PEG-lipid functionalized CPNs as a fluid phase marker in J774A.1 cells compared to cells loaded with carboxy-functionalized quantum dots (Q dots) or a dextran–linked small molecule organic dyes (Alexa fluor 488 dextran (AF488-dex)). Under typical conditions used for ex vivo biological imaging or flow cytometry, these CPNs were 175x brighter than Qdots and 1,400x brighter than AF488-dex. Evaluation of the minimum incubation concentration required for detection of nanoparticle fluorescence with a commercial flow cytometer indicated that the limit of detection for PEG lipid-PFBT CPNs was 19 pM (86 ppb), substantially lower than values obtained for Q dots (980 pM) or AF488-dex (11.2 nM). Taken together, these data clearly indicate that CPNs can be used at very low labeling concentrations and are excellent fluid-phase markers with significantly greater fluorescence brightness than existing dyes or nanoparticles.
Chapter 4 studies the protein passivation of CPNs as a reliable approach to provide the colloidal stability of conjugated polymer nanoparticles in tissue culture media and buffer solutions. Unmodified CPNs aggregate under physiological salt conditions and are therefore unsuitable for biological applications such as imaging and sensing. We showed that when incubated in protein solutions (bovine albumin serum, lysozyme, or fetal bovine serum), bare CPNs rapidly acquire a stable protein coat that both increases CPN diameter and prevents aggregation at physiological ionic strength over pH range of 4 - 8. The protein coat is highly stable; no change in hydrodynamic diameter is observed upon extended incubation following size exclusion chromatography into protein free saline solution. The results show that adsorption of protein on CPN surface does not alter fluorescence brightness. BSA-biotin modified CPNs show availability of protein corona for molecular recognition. Hence, we concluded that protein adsorption is a simple method to provide colloidal stability in physiological buffer and to modify CPNs for target selective cellular imaging and sensing.

Finally, Chapter 5 reports the study of protein coating as a general method for providing functionality in CPNs. We have demonstrated that protein-A coated CPNs serve as scaffold for CPN linkage to IgG. Using anti-rabbit IgG linked CPNs as a detection reagent; we have detected rabbit IgG in solid phase immunoassay with antigen-antibody binding constant of 5.5 ± 0.8 nM. Similarly, neutravidin coated CPNs that conjugated to biotin linked recognition moiety also serve as a direct detection reagent. Together, we conclude that protein A, neutravidin and immunoglobulin modified CPNs serve as direct detection reagent in solid phase based immunoassays.
Taken together, this study shows that head group functionalized phospholipids and a broad variety of proteins readily modify the surface of hydrophobic conjugated polymer nanoparticles. The resulting CPNs retain and improve fluorescence brightness. Hence, head group functionalized phospholipids and proteins act as linkers for biomolecules on the nanoparticle surface. Such CPNs are bright photon source for specific labeling, imaging, and sensing applications.
DEDICATION

This dissertation is dedicated to my parents, Mani Raj and Tika Maya Kandel for all their love and encouragement. They have been supportive in every possible way throughout my life. It is also dedicated to all of my family and friends who have accompanied me through and helped me along this journey.
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CHAPTER ONE
INTRODUCTION

Conjugated polymer nanoparticles (CPNs) have attracted attention as promising fluorescent nanoparticles because of their high fluorescence brightness. They are prepared by precipitation of hydrophobic fluorescent conjugated polymers that exhibit bright fluorescence as nanoparticles. These nanoparticles possess the brightest fluorescence per unit size of all characterized nanoparticles [1]. Unlike many other nanoparticles, these consist of relatively benign polymers; therefore, they do not have observable cytotoxicity [2-4]. Their high brightness, reasonable photostability, and no observable cytotoxicity consequently provide a motivation for studying conjugated polymer nanoparticles (CPNs) as a fluorescent label. These CPNs have found applications in fluorescence based experiments such as cellular labeling, imaging, single particle tracking, and biosensing [1, 3, 4, 6].

To realize the potential for biological applications of CPNs, it is required that they possess functionality on their surface and maintain colloidal stability in biological media. Presence of a functional group on the nanoparticle surface allows conjugation of recognition moiety which is necessary for specific detection and targeted labeling. However, the CPNs prepared from hydrophobic conjugated polymer by reprecipitation in water lack a functional group and tend to aggregate in high ionic strength salt solution [6, 7]. This limits the wide use of CPNs in biological applications. In this context, we have investigated approaches to functionalize the predominantly hydrophobic CPN surface and
also provide colloidal stability in biological media. Since the fluorescence properties of conjugated polymers arise from a delocalized \(\pi\)-electron system, chemical modification to introduce covalent functionality can destroy the delocalized electronic system causing potentially unintended changes in optical properties. Given the hydrophobic nature of CPNs, non-covalent interactions with biomolecules readily occur. Moreover, key interactions in molecular recognition processes involve non-covalent interactions. In this dissertation, we have modified and functionalized conjugated polymer nanoparticles (CPNs) by surface passivation with head group-functionalized poly (ethylene glycol) lipids and a variety of relevant proteins. We demonstrated that CPNs functionalized with a biotin end group PEG lipid can be utilized for targeted cell surface labeling and imaging. We studied the colloidal stability of surface modified CPNs in biological media and investigated their utility as fluid phase label in cellular imaging and flow cytometry experiments. In addition, we demonstrated that the CPNs decorated with a protein corona can be used as direct detection reagents in immunoassay.

1.1 CONJUGATED POLYMER AS NANOPARTICLES

Since the discovery of conducting polymers and the ability to control electrical conductivity by doping, conjugated polymers have been characterized and their properties investigated [8, 9]. They have widely been used as active materials in the light emitting diodes [10-12], photovoltaic devices [13-16], and thin film transistors [17, 18]. The preparation and properties of conjugated polymers, as well as its application in solution and thin films, have been extensively studied [13, 14, 19, 20]. However, by
comparison, there have been relatively few studies of conjugated polymers in the form of colloidal dispersion of nanoparticles. In particular, CPNs have been targets of investigation as fluorescent labels in biological applications [5].

Beginning in the 1980s, Vincent et al reported colloids of conducting polymer nanoparticles produced by aqueous oxidative polymerization of acetylene [21] and polypyrrole [22]. Since then, various approaches have been employed to access nanoparticle colloids in water. Principally, depending on molecular mass of starting materials, conjugated polymer nanoparticles can be synthesized by two different approaches. In the direct polymerization approach, nanoparticles are synthesized from low molecular weight monomers in a heterophase system. In addition, polymers entirely insoluble in any solvent can be converted into nanoparticles by this method. Vincent et al, also reported the preparation of polyacetylene particles by polymerizing acetylene in the presence of steric stabilizers [21]. Recently, this method has been adopted to access nanoparticles from a fluorescent semiconducting polymer [23-26]. The other common method utilizes a post polymerization approach. In this method, nanoparticles are prepared from high molecular weight polymers which have already been synthesized and purified. This type of nanoparticle synthesis relies on off-the-shelf commercially available conjugated polymers and does not require polymer synthesis expertise. The post polymerization approach is a relatively simple method and the preferred method of nanoparticles formation from fluorescent conjugated polymers by non-organic chemists. There are the general subcategories of the post-polymerization method which mainly include miniemulsion and reprecipitation. Typically, these methods of nanoparticle
preparation involve pre-synthesized conjugated polymer dissolved in organic solvent as a starting solution. Nanoparticle formation is carried out either by creating emulsified droplets in a water immiscible solvent and subsequent solvent removal (miniemulsion) or rapidly mixing polymer solutions during rapid dilution in an excess of solvent that is miscible with organic solvent most commonly water (reprecipitation).

1.1.1 REPRECIPITATION

The reprecipitation method involves mixing of a very dilute polymer solution dissolved in organic solvent, for example, tetrahydrofuran, into an excess of poor polymer solvent (water). This process is aided by sonication to assist the formation of nanoparticles. Mixing of the conjugated polymer dissolved in good solvent directly into poor solvent results in sudden alteration of solvent environment that induces precipitation of polymer. After precipitation into water, the organic solvent is evaporated at elevated temperature under vacuum resulting in nanoparticle dispersion in water. This preparation method can be used for wide variety of hydrophobic conjugated polymers that are soluble in water miscible organic solvents.

Mashura et al used the reprecipitation method to prepare substituted-polythiophene particles by mixing a polymer solution in tetrahydrofuran (THF) into water. The resulting particle diameters range from 40-400 nm when measured by dynamic light scattering (DLS) [27]. This method was adopted and modified by McNeill et al to produce nanoparticles of smaller sizes from hydrophobic conjugated polymers [28-35]. In this procedure, a very dilute solution of conjugated polymer dissolved in organic solvent such
as THF or DMSO is rapidly mixed in water during sonication. The sudden change from a good polymer solvent to a poor polymer solvent alters solubility of polymer causing polymer to precipitate into dispersion of nanoparticles. The polymer chain in nanoparticle adopts a collapsed conformation and forms spherical nanoparticles [36]. The mechanism of the colloidal stability of hydrophobic polymer particles in water with no surfactant is not clear; however, Clafton et al shows evidence of the presence of negatively charged surface defects on conjugated polymers during the formation of nanoparticles. Negative charge on the nanoparticle surface, as consequence, provides electrostatic stabilization to colloidal solution in water [37]. The average particle size prepared from this method ranges from 5-50 nm. The particle size is generally controlled by adjusting precursor polymer concentration and polymer composition. Compared to the miniemulsion technique, precipitation method generally yields smaller nanoparticles. In some cases, the small particle size corresponds to single polymer chain particles [36, 38].

1.2 DEVELOPMENT OF FLUORESCENT CONJUGATED POLYMER NANOPARTICLES

Fluorescence spectroscopic techniques in combination with improved fluorophores are an important tool for lowering the limit of detection in biological experiments. In particular, fluorophores sufficiently bright and with stable fluorescence allow investigation of biomolecular interactions at single molecule level which can provide spectacular details of biochemical processes [39]. In addition, the combination of fluorescence spectroscopy and imaging techniques enables researchers to make
measurements in multiple spatial and temporal dimensions. Traditional fluorophores in biological imaging and bioassays include small molecule fluorescent dyes such as fluorescein, rhodamine, and cyanine [40]. However, low absorptivity and poor photostability of conventional dyes limits the development of high sensitivity cellular imaging and in vitro assays especially at the single molecule level. Therefore, there is a need to develop fluorophores to maximize sensitivity and minimize limit of detection. Such nanoparticles include inorganic quantum dots (QDs) [41, 42], dye doped silica particles [43] and commercially available dye-loaded latex spheres. These nanoparticles offer numerous advantages over traditional organic dyes, including bright fluorescence and improved photostability. However, QDs, despite their superior optical properties with respect to organic fluorophore dyes, can suffer from problems of cytotoxicity due to leached metal from the nanocrystal core [44, 45] and the existence of dark dots [46]. Heavy metal leaching has been reduced by coating QDs with a variety of materials; however, such coatings can have their own associated cytotoxic effects [44, 47]. Fluorescent dye loaded polymer latex or silica nanoparticles also exhibit improved brightness and photostability than molecular dyes because of the large number of fluorophores per particle [48-50]. But, self-quenching of dye when loaded at high concentration ultimately limits the overall brightness of such particles [43].  

In this context, the limitations of current fluorescent nanoparticles provide motivation for the development of other classes of nanoparticles with high photostability and bright fluorescence, but with reduced cytotoxicity. Great efforts have been invested to design and prepare nanoparticles that can overcome the limitations of existing
fluorophore and exhibit bright fluorescence, high photostability, and no cytotoxicity for biological applications. To that end, the development of CPNs is one promising strategy. CPNs are extraordinarily bright because of high absorption cross-sections and high radiative rates [29, 51]. The nanoparticles are composed of relatively benign constituent polymer material. As a result, there is low or no observed cytotoxicity [2]. These merits established CPNs as bright fluorescence probes for improving sensitivity and limit of detection in biological applications.

For biological applications, the nanoparticles composed of conjugated polymers exist in many different forms. Nanoparticles prepared from hydrophobic conjugated polymer by reprecipitation method in water are the most common type [29-35, 52-57]. Water as a dispersion medium is preferred for most biological applications. This method generates nanoparticles with diameter in the range of 10-30 nm which can be adjusted by varying the polymer concentration in the stock solution in organic solvent [58]. Competition between inter chain aggregation and intra chain collapse during the nanoparticle formation process dictates the size of the nanoparticles. Preparation of particles from dilute stock solution reduces the possibility of inter chain aggregation that results smaller particles. The CPNs composed of hydrophobic polymers in water is thought to assume a collapsed spherical structure due to strong hydrophobic interaction and densely packed chromophores [59-61].

Not only hydrophobic conjugated polymers but also hydrophilic conjugated polymers and conjugated polyelectrolytes can be converted into nanoparticles [4, 62-66]. Poly (p-phenylene ethynylene), PPE polymer containing a hydrophilic amine and a PEG
linker in dimethylsulphoxide (DMSO) was mixed into saline solution to generate nanoparticles of much larger size (ca. 500 nm)[62]. The same group prepared nanoparticles of smaller size (ca. 97 nm) using similar hydrophilic polymers following method optimization and purification by sequential ultrafiltration. Polyelectrolytes are converted into nanoparticles by self-assembly and can have an average hydrodynamic particle size of about 80 nm [67, 68]. These CPNs consists of loosely aggregated polymer chains in comparison with hydrophobic CPNs which have a densely packed structure. Similarly, fluorescent amphiphilic polymers based on hydrophobic polyfluorene backbone containing hydrophilic PEGs on side chains form nanoparticles in water. Slow addition of water to a solution of polymer in THF results in micellar nanoparticles [69]. The size of particles prepared from amphiphilic polymers ranges from 10 nm to 100 nm [70-73]. Such nanoparticles have been used for cellular labeling and imaging applications.

1.3 BIOLOGICAL APPLICATIONS OF CONJUGATED POLYMER NANOPARTICLES (CPNs)

The useful photophysical features including high brightness, photostability, and low cytotoxicity is promising for a wide range of biological applications using CPNs. Generally, these nanoparticles find applications in biological studies such as: (a) labeling, imaging and tracking (b) sensing and assay readout, and (c) gene and drug delivery. All varieties of CPNs based on hydrophobic polymers, polyelectrolytes, or amphiphilic polymers have been employed for imaging and sensing applications.
For biological applications, biocompatibility of nanoparticles is an important concern. The utility of a given probe for biological applications in live cells is compromised if the probe causes cell death or other deleterious effects. Cytotoxicity of conjugated nanoparticles has been studied by several groups and all studies show very low or no cytotoxicity of CPNs. This observation is attributed to the benign nature of conjugated polymer which is the major constituent of nanoparticles. Fernando et al studied possible cytotoxicity and inflammatory responses of hydrophobic conjugated polymer nanoparticles (ca. 18 nm) in J774A.1 cells. Cell viability was assessed for (poly [(9,9-dioctylfluorenyl-2,7-diyl)-co-(1,4-benzo-{2,1',3}-thiadiazole)], PFBT CPNs loaded macrophage cells with Cell Titer Blue, a dye which tracks cell viability and cell proliferation. The percentage of viable J774A.1 cells incubated with CPNs for 18 hours is indistinguishable from cells incubated without CPNs at all concentrations tested supporting the claim of the benign nature of CPNs. To evaluate the likelihood of a CPN induced inflammatory response; expression of the proinflammatory cytokines, tumor necrosis factor α (TNFα) and interlukin-1β (IL-1β) at the mRNA level were monitored in cells with and without nanoparticles. The results suggest that these CPNs have no inflammatory effects in this type of cells [2]. The same group studied cytotoxic effects of PEG-lipid incorporated CPNs by monitoring cell viability with Cell Titre Blue. They show that even at the CPN concentration 25 fold higher than the working concentration used for cell labeling; there is no discernible impact in cell viability [74]. Moon et al investigated the cytotoxic effects of nanoparticles prepared from hydrophilic poly (p-phenylene ethynylene polymer derivatives in baby hamster kidney (BHK) cell lines [4].
The cells incubated with ca. 97 nm particles and live cells were quantified at varying times using Cell Titre-Glo assay kit, which measures ATP to determine the number of viable cells. Results show that there is minimal inhibitory effect of nanoparticles in cell viability over a course of one week. Liu et al evaluated cytotoxicity of fluorescent conjugated polymer loaded poly (DL-lactide-co-glycolide), PGLA, nanoparticles larger size (ca. 220 nm) in NIH/3T3 fibroblast cells. The metabolic viability assay of NIH/3T3 cells incubated with poly [9,9-bis(2-(2-(2-methoxyethoxy)ethoxy)ethyl)fluorenlyl-divinylene], loaded nanoparticles at various PFV concentrations indicated the low cytotoxicity of these CPNs. They did not observe effects in metabolic activity even at 700 nM PFV concentration for two days [75]. These results suggest that CPNs have low toxicity to the cells and suitable for cellular labeling and imaging for extended periods.

1.3.1 CELLULAR LABELING

Wu et al utilized multicolor conjugated polymer nanoparticles for cellular imaging in macrophage cells. These experiments involved the labeling of cells using bare hydrophobic conjugated polymer nanoparticles which were efficiently taken up by cells via endocytosis [1]. Fernando et al studied the mechanism of cellular uptake of non-capsulated bare PFBT nanoparticles in macrophage cells using 18 nm diameter CPNs. The nanoparticles could be detected at extremely low loading concentration of 155 pM (270 ppb). Intracellular co-localization studies of CPNs and Texas red dextran indicated that cellular uptake took place through a macropinocytic mechanism. Again, CPNs and anti-LAMP-1 antibody staining indicated that the final destination of nanoparticles was
lysosomes following particles internalization via endocytosis [57]. Macropinocytosis is a nonspecific mechanism for taking up extracellular fluid which does not recognize and discriminate nanoparticle surface and charge delivering the fluid content to lysosome. This mechanism is useful in fluid phase labeling of endosomal and lysosomal organelles.

In another study, PLGA nanoparticles loaded with conjugated polymer have been used for cellular labeling. When incubated with nanoparticles, MCF-7 breast cancer cells internalized these larger particles and were delivered into the cytoplasm [76]. The same group utilized folic acid conjugated particles to specifically target the folate receptor in cultured cancer cells. They reported the improved uptake of folic acid functionalized particles in MCF cancer cells than the non-functionalized ones by MCF cancer cells through folate receptor mediated endocytosis. Similarly, conjugated polyelectrolyte nanoparticles and amphiphilic conjugated polymer nanoparticles have also been demonstrated to be an effective cellular label [4, 73, 77-79]. Cellular uptake of nanoparticles was dependent on functional surface groups, the specific cell lines, and mainly occurred through both macropinocytosis and receptor mediated endocytosis.

Endocytosis-based cellular labeling methods lack specificity for targeted labeling. The targeted labeling method generally involves antigen-antibody interactions and affinity of receptor-ligand system such as streptavidin-biotin. To develop CPN probes for immunofluorescent labeling, Chiu et al developed a bioconjugation method with carboxy functionalized CPNs. A carboxy functional group was introduced in CPNs by blending an amphiphilic polymer, containing carboxy end groups and subsequent linking of antibody or streptavidin based on standard conjugation chemistry [80]. Using this
probe, they labeled a specific cellular target, EpCAM, which is an epithelial cell surface marker present in circulating tumor cells. The results demonstrate the specific and effective labeling of targets. In addition, they compared the brightness of the CPNs labeled live MCF-7 cells against Q dot 565 labeled cells and Alexa 488 labeled cells using flow cytometry. Quantitative analyses of fluorescence intensity of these labeled cells shows that these CPN labeled cells were ca. 25 times brighter than that of Qdots labeled cells and ca. 18 times brighter than that of Alexa dye labeled cells. This indicates the potential of CPNs for cellular imaging and fluorescence based biological detection. The same group reported the smaller CPN of ca. 10 nm diameter bright orange fluorescence from the cyano substituted poly (p-phenylenevinylene) polymer. These probes were used in specific labeling of a cell surface marker and microtubule structures in HeLa cells by immunofluorescence [81].

1.3.2 IN VIVO IMAGING

Fluorescence imaging has the potential for a wide variety of molecular diagnostics and therapeutic applications. Because of its high sensitivity, fluorescence based imaging techniques are being increasingly utilized in small animal research. However, photon limiting interferences such as scattering, absorption, and autofluorescence limits their ability to generate effective deep-tissue fluorescence imaging. To overcome these limitations, near infrared (NIR) fluorescence probes with fluorescence, an order of magnitude higher than that of currently used organic dyes, is desired. To that end, conjugated polymer nanoparticles hold the potential to overcome the
challenges because of their extreme fluorescence brightness. Kim et al used a cyano-substituted derivative of poly (phenylenevinylene) to prepare nanoparticles that emit in the red to NIR region. They demonstrated the in vivo fluorescence mapping of sentinel node (SNL) in mouse model using nanoparticles of 60±14 nm in size[82]. Similarly, Chiu et al developed conjugated polymer nanoparticle of ca. 15 nm using a conjugated polymer blend with deep red emission (λ= 655nm). The probe was functionalized and conjugated to chlorotoxin which is a tumor specific peptide ligand for in vivo tumor targeting. Biophotonic imaging and quantitative analysis of bio-distribution demonstrates that these probes have the ability to target malignant brain tumors specifically [83].

1.3.3 SENSORS

FRET (Fluorescence resonance energy transfer) based techniques allows the detection of receptor-ligand interactions and biomolecular conformational changes in response to binding [84-86] and provides the basis for FRET based sensing applications. Conjugated polymer electrolytes have been employed as sensitive FRET-based bio- and chemical sensors [68, 87, 88]. Wu and coworkers have developed a sensing system based on energy transfer between components doped inside hydrophobic polymer nanoparticles. The sensing moieties for specific analyte are incorporated into the hydrophobic nanoparticle. The intraparticle energy transfer from matrix polymer donor to the entrapped dye molecule results in emission that is sensitive to specific analyte or responsive to changes in the local environment.
An oxygen sensor was developed by doping a blue emitting conjugated polymer with a phosphorescent dye, platinum octaethylporphyrin (PtOEP), which is responsive to molecular oxygen [89]. This ratiometric single nanoparticle oxygen sensor exhibited energy transfer from conjugated polymer host to the dye molecule. As the oxygen content decreases, phosphorescence of the oxygen sensitive dye increases making it a useful ratiometric sensor to monitor cellular hypoxia. Based on the same strategy of incorporating fluorescent dyes, other conjugated polymer based sensors were developed. For example, a pH sensitive probe was developed by covalently linking hydrophilic fluorescein dye to conjugated polymer poly (p-phenylenevinylene) surface [90]. The energy transfer from polymer donor to fluorescein acceptor makes it ratiometric pH sensor. Intracellular pH was measured between 5.0 and pH 8.0 after delivering the probe into HeLa cells by endocytosis. Similarly, temperature sensitive conjugated polymer nanoparticles were developed by incorporating the temperature sensitive dye Rhodamine B (RhB) in the conjugated polymers PFBT and PFPV [91]. A hydrophobic polystyrene polymer linked covalently with hydrophilic Rhodamine B dye was mixed with conjugated polymers and converted into nanoparticles by precipitation. This ratiometric sensor where polymer matrix transfers energy to dye molecule shows a linear fluorescent response from 10°C to 70°C.

The same group has extended the idea to develop ion sensors to detect biologically relevant ions such as Cu²⁺, Fe²⁺ based on fluorescence quenching by aggregation induced by these ions. The Cu²⁺ induced aggregation is reversible upon the addition of ethylenediaminetetraacetic acid (EDTA) and fluorescence is recovered. Fe²⁺
induced aggregation is not reversible and the fluorescence remains quenched. This differential in fluorescence allows the identification of copper and iron ions and the determination of specific species concentrations [92]. Harbron et al developed polymer nanoparticle sensors to detect mercury in aqueous solution by doping the molecular dye rhodamine spirolactam (RhB-SL), which is nonfluorescent and becomes fluorescent when exposed to mercury ions [93]. The fluorescent intensity ratio of the dye acceptor to PFBT donor allows the ratiometric sensing of mercury. These efforts indicate that CPNs show promise for chemical and biochemical sensing applications.

1.4 RECENT FUNCTIONALIZATION STRATEGIES

Hydrophobic conjugated polymer nanoparticles prepared by reprecipitation method in water lack functional surface properties. Functionality on nanoparticle surface allows conjugation to useful biomolecules required for specific labeling, targeted delivery, and sensitive detection. In addition, surface modified nanoparticles enhance solubility in water and improve colloidal stability to otherwise hydrophobic nanoparticles. Recently, various approaches have been employed to address this problem and several research groups are involved in developing effective functionalization methods. Initially, surface functionalization strategies of quantum dots were adopted to functionalize CPN surface. For example, encapsulation is a general strategy utilized in the functionalization of nanoparticles. Functionality in conjugated polymer nanoparticles has been introduced by encapsulating CPNs in functional materials such as silica [94], phospholipids [7, 95, 96] and PGLA polymers [76, 97].
Silica encapsulation has been widely used to functionalize nanoparticle surface [49, 98, 99]. This method has produced nanoparticles of 20-30 nm in diameter with few nanometers of silica that provides functionality for specific labeling of biomolecules. Utilizing the same strategy, Wu et al encapsulated the conjugated polymer nanoparticles of size 10-20 nm with 2-3 nm thick silica shell to incorporate functionality [94]. The silica encapsulation method can yield relatively small particle sizes, but the likelihood of hydrolysis of the silica shell causes leaching of encapsulated fluorophores in biological environments. In another approach to encapsulate hydrophobic conjugated polymer in phospholipid, Green et al adopted a strategy of encapsulation of quantum dots in phospholipid using the miniemulsion method [95,100]. The average diameter of the encapsulated nanoparticles in water ranges from 80 nm-100 nm. Incorporation of PEG-lipid-COOH in nanoparticle during the encapsulation process results in carboxy functionalized nanoparticles that allowed conjugation of BSA. Employing the same strategy, both iron oxide nanoparticles and conjugated polymer were encapsulated inside phospholipid micelles [96]. To demonstrate their potential as a bimodal imaging probe, magnetic resonance imaging (MRI) was performed with the particle suspension. In another encapsulation approach, Li et al used PLGA with the aim to utilize the -COOH functional group of the matrix as a handle to conjugate biomolecules. The PLGA nanoparticles loaded with conjugated polymers prepared by the miniemulsion method have been used for targeted cellular labeling [76, 97]. The hydrodynamic diameter of PLGA particles loaded with conjugated polymer as measured with dynamic light scattering (DLS) are larger (243 – 272) nm. The relatively larger size for PLGA and
phospholipid encapsulated particles is the limiting factor in many cellular and subcellular targeting applications. Moreover, the low fluorophore loading concentration in the nanoparticles prepared by the encapsulation method prevents the formation of densely packed fluorophore polymer nanoparticles. This consequently decreases the per particle brightness of the nanoparticles. Therefore, an alternative approach to prepare nanoparticle with densely packed chromophore is desirable.

CPNs prepared through the reprecipitation method are relatively small in size and contain a high volume fraction of conjugated polymer which results in high per unit size brightness. This is an important factor for cellular imaging applications. However, incorporation of a functional surface is crucial for bioconjugation and specific labeling of cellular targets. To that end, Chiu et al developed various strategies for introducing functional moieties in CPNs (Pdots) for bioconjugation [61,101-105]. The first strategy is based on intertwining and trapping amphiphilic polymer chains bearing functional groups inside CPNs during the nanoparticle formation process. Incorporation of a functional amphiphilic polymer modifies the nanoparticle surface offering a handle to link biomolecules to nanoparticles. Cellular target labeling is carried out based on specific biomolecular interactions such as antigen-antibody or biotin-streptavidin interactions. Functionalization by trapping functional polymers and subsequent bioconjugation strategy are applicable to any hydrophobic fluorescent conjugated polymer. For example, carboxy functional group was introduced in PFBT nanoparticle surface using an amphiphilic comblike polystyrene polymer, PS-PEG-COOH at various fractions [101]. Size measurement by TEM and DLS shows the average diameter of nanoparticle is
15 nm. Functionalized nanoparticles prepared from this strategy contain 80% effective fluorophores and 20% PS-PEG-COOH. Compared to encapsulation approaches where concentration of effective chromophore in nanoparticle is low, this strategy produces particles with high fluorophore density. The available carboxy functional group is utilized to conjugate biomolecules using standard conjugation chemistry such as carbodimide crosslinking. In another study to utilize click chemistry for cellular labeling, carboxy functionalized nanoparticles were prepared by coprecipitating the fluorescent PFBT polymer blend with a small amount of functional polymer, poly (styrene-co-maleic anhydride; PSMA)[102]. Utilizing carbodiimide conjugation chemistry, the carboxy functionalized Pdots were linked with amine group of a small molecule such as amino azides and amino alkynes. Biomolecules were labeled using copper (I) catalyzed click chemistry. These are examples of carboxy functionalization and subsequent bioconjugation of CPNs for cellular labeling experiments.

However, incorporation of functional group by encapsulation and intertwining amphiphilic polymer with conjugated polymer by coprecipitation relies on non-covalent hydrophobic association. Disassociation of functional moiety as a result of swelling in high ionic strength solution or unfolding of polymer structure causes decrease in functional group carrier. So, in an another approach, Chiu et al employed a synthetic approach to introduce covalently linked functional group in polymer side chain before converting into nanoparticle [60]. Nanoparticles prepared from such polymer allow direct functionalization without the additional step of surface modification procedures. However, such direct functionalization requires modification of polymer side chains
demanding expertise in polymer synthesis. Also, the density of hydrophilic functional
group plays an important role in the nanoparticle compactness, stability and fluorescence
performance in solution [60]. They investigated the effect of varying hydrophilic side
chains on the nature of nanoparticles formed and their optical performance. To study this
effect of modification, the PFBT polymer with side chain COOH at molar fraction of 2%,
14% and 50% were synthesized. As the density of –COOH side chains increased, the
quantum yield of the respective nanoparticles decreased. This is the result of less compact
nanoparticles with increasing hydrophilicity within the polymer chain. As a variant of the
direct functionalization approach, the same group developed a method to synthesize
PFBT polymer with imine group side chains and cross linked them with a functional
polymer such as poly (isobutylene-alt-maleic anhydride) PIMA, which contains multiple
reactive units. The functionalized polymer is converted into nanoparticles that bear
carboxy functional groups on surface for further bioconjugation [61]. The particle size of
the functionalized CNPs is ca. 10 nm which was successfully conjugated with
streptavidin. Cancer cells were labeled with this probe and biological imaging was
performed.

1.5 INTERACTION OF PROTEINS WITH NANOPARTICLES

Protein adsorption on the surface of colloidal particles occurs when they come in
contact with protein solutions. Studies on protein and antibody adsorption on varieties of
microparticles composed of glass [106], aluminosilicate[107], polyanhydride[108] and
polystyrene latex particles[109] have shown that protein bound on the particle surface is
irreversible and adsorption depends on particle size, charge and hydrophobicity. Similarly, adsorption of proteins on the nanoparticle surface has also been studied for various types of nanoparticles that demonstrate irreversible protein binding on the surface [110-112]. Investigations into the pattern of protein adsorption on nanoparticles indicates that the protein corona composition changes as a function of nanoparticle type, size, surface properties and curvature of nanoparticles [113, 115-117]. The protein binding on nanoparticle surfaces includes an irreversibly bound inner layer called the hard corona and reversibly bound external layer called the soft corona [114]. Using the model system of adsorption of transferrin to sulfonate polystyrene nanoparticles and carboxyl-polystyrene nanoparticles, Milani et al showed that a strongly bound first monolayer forms followed by weakly bound secondary layer around it which depends on molar ratio of protein to nanoparticles. The hard corona is stable and remains associated with nanoparticles even after removal of free protein. Varieties of proteins that are known to associate with nanomaterials have been studied by various researchers and have identified hard protein corona around nanoparticles [111]. Protein composition in the nanoparticle environment and protein concentrations also plays a role in the corona composition [118-121]. Generally, the thickness of protein corona increases with increasing hydrophobicity, charge density and curvature of nanoparticles [111].

Varieties of nanoparticles have been functionalized with biomolecules such as peptides, proteins, enzymes and nucleic acids to make the use of these nanoparticles. Generally, the association of biomolecules on the nanoparticle surface occurs by nonspecific chemisorption. Such nonspecific adsorption occurs by electrostatic attraction,
van der Waals forces or hydrogen bonding when nanoparticles are incubated with or comes in contact with the proteins and are irreversibly adsorbed [125-127]. For example, such adsorption processes not only provide colloidal stability but also introduces functionality for molecular recognition in quantum dots nanoparticles [122-124]. Proteins that come into intimate contact with the nanoparticle surface causes partial or complete denaturation [128] and the presence of protein on particle surface affects biological activity. For a series of nanoparticle types, including polystyrene, iron oxide and silica nanoparticles, cellular uptake and cytotoxicity is reduced by a protein coating[110,129,130] while other nanoparticles show increased uptake in the presence of protein corona[131]. These data indicate that the recognition surface presented to cells by the protein coat determines the efficiency of cellular uptake rather than the nanoparticle core characteristics [132].

In order to provide an inert, biocompatible and hydrophilic surface coating to nanoparticles, polyethylene glycol (PEG) has been used as a surfactant. PEG modification provides colloidal stability of nanoparticles in high ionic strength solution and in biological media [133,134]. Functional end group PEG molecules not only provide steric stabilization to nanoparticles but also provide functional groups to nanoparticle surface. Surface properties of encapsulated nanoparticles depend on the coating materials rather than material on the particle core [135-136]. Structural characterization of PEG lipid coated drug nanocarriers show that PEG lipids are firmly attached to the surfaces of nanoparticles [137]. Therefore, surface passivation of hydrophobic conjugated polymer nanoparticles by head-group functionalized PEG-lipids is a strategy to provide functional
biomolecular conjugation and colloidal stability. Similarly, the evidence of protein adsorption on varieties of nanoparticle surfaces and formation of irreversible hard coronas offers another method to provide functionality in CPNs for biological applications including immunoassay.

In this dissertation, we demonstrated a simple method for passivation and functionalization of CPNs by incorporating head group modified PEG-Lipids and proteins. This method allows modification of otherwise hydrophobic surface and provides colloidal stability in biological media. In addition, incorporation of functional phospholipids and protein adsorption on the nanoparticle surface offer methods to provide the CPN surface with molecular recognition and targeting moieties. We have shown that surface modified particles designed to lack biological functionalities are taken up efficiently by macrophage cells via micropinocytosis. Fluorophore loaded cells showed that CPNs are brighter fluid phase markers compared to dextran-linked alexa fluor organic dye and carboxy functionalized quantum dots. We have also studied the protein and antibody adsorption on nanoparticles surface which shows that hard protein corona on nanoparticle surface makes it a direct detection reagent in immunoassay. Taken together, we conclude that the utility of such particles ranges from targeted cellular labeling, fluid phase markers, and direct detection reagents for biomolecular methods.
1.6 REFERENCES


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CHAPTER TWO

INCORPORATING FUNCTIONALIZED POLYETHYLENE GLYCOL LIPIDS INTO REPRECIPITATED CONJUGATED POLYMER NANOPARTICLES FOR BIOCONJUGATION AND TARGETED LABELING OF CELLS

2.1 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 particles [3], 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 that include biosensing, live cell imaging, and intracellular dynamics [4, 6]. 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 nanoparticles [3] 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 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 with intrinsic fluorescence, they have low cytotoxicity [13], and cannot leach dye or constituent materials. As a result, CPNs have established themselves as useful optical probes that can be used at extremely low concentrations. 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. In addition, this category of nanoparticle has not previously been conjugated to useful biomolecules for targeted delivery to cells.

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. Another strategy is to envelope the CPNs with hydrophilic component(s), which would alter the nanoparticle surface characteristics without changing polymer optical qualities [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 nanosensor [16], to polymer coated quantum dots [17-20] and to
semiconductor polymer nanospheres formed by miniemulsion and also referred to as semiconductor polymer nanospheres [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 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 the straightforward reprecipitation method to prepare highly fluorescent CPNs that incorporate functionalized PEG lipids, using commercially available materials. 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, possibly including applications with single nanoparticle detection limits.
2.2 METHODS AND MATERIALS

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-divinylenefluorenylene)-alt-co-{2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene}]), MW 85,000, polydispersity 5.4), MEHPPV (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). Mr 2000 PEG lipids with biotin end groups (1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N-[biotinyl (polyethylene glycol)-2000];(ammonium salt)), and carboxy end groups (1,2-dimyristoyl-sn-glycerol-3-phosphoethanolamine-N-[carboxy (polyethylene glycol)-2000];(ammonium salt)and 550,1000, and 2000 M, PEG lipid with methoxy end group (1,2-dimyristoyl-sn-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.

2.2.1 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 M r PEG). 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 and 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 polyvinylidene fluoride (PVDF) syringe filter (National Scientific).

2.2.2 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 were 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 second 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 to 700 nm in aqueous solution using 460 nm excitation. PFO nanoparticles were excited at 384 nm and emission measured from 395 to 700 nm. PFPV nanoparticles were excited at 458 nm and emission measured from 480 to 700 nm. MEH-PPV nanoparticles were excited at 498 nm and emission measured from 510 to 725 nm. 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 resolutions 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 solutions assuming complete polymer to nanoparticle conversion. Specifically, nanoparticle volumes were calculated from the particle diameter measured by TEM, assuming a spherical shape and converted to nanoparticle mass assuming a nanoparticle density of 1 g cm$^{-3}$ (actual density is between 0.95 and 1.05 g cm$^{-3}$); 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 any loss from filtration, if any, is small, and demonstrate negligible formation of aggregates.

2.2.3 CONCENTRATING PEG LIPID MODIFIED NANOPARTICLES

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

2.2.4 STREPTAVIDIN PULL-DOWN OF BIOTIN PEG LIPID-PFBT NANOPARTICLES

PFBT nanoparticles (28ppm) prepared with biotin functionalized PEG lipid were incubated with magnetic streptavidin beads (New England Biolabs) for 30 min in phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4). The magnetic beads were removed from solutions using a strong permanent magnet and washed 5x in PBS to remove any unbound CPNs. The magnetic beads were then incubated overnight with 0.2 mg ml⁻¹ free biotin to dissociate the CPNs from the magnetic beads. The magnetic beads were then removed from solution using a strong permanent magnet. The fluorescence from the remaining biotinylated nanoparticles was
measured at 460 nm excitation while scanning the fluorescence emission from 480 nm to 700 nm. The slits for both the excitation and emission monochromators were set to achieve a 2 nm band pass.

2.2.5 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 air. Clean cover glasses then coated with 1% poly-L-lysine, washed with water to remove excess poly-L-lysine, incubated with 1 mg ml\(^{-1}\) streptavidin for 30 minutes, and carefully washed with Ringer’s buffer 3x (RB; 155 mM NaCl, 5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 2mM NaH\(_2\)PO\(_4\), 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 imagine 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).
2.2.6 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 ºC). Adherent cells were washed with RB 3x, fixed with 4% paraformaldehyde for 10 minutes at 37 ºC 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 1µg ml⁻¹ 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 Mr PEG lipid-PFBT or carboxy 2000 Mr 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 (Hamamatus)]. The acquired images were analyzed using Slidebook 5.0.
2.3 RESULTS AND DISCUSSION

2.3.1 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 PEG lipid, during brief mild sonication to aid mixing, as described in detail in the experimental section. The PEG lipid molecules used here contain two C_{14} lipid chains linked to the PEG through a phosphate moiety and provide a bidentate hydrophobic group for interaction with conjugated polymer. The functional end group is located at the opposite end of the PEG chain.

PEG lipid-CPNs were prepared using PFBT and a series of PEG lipids (PEG Mr = 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. Different end groups provide different moieties for conjugation to biomolecules; carboxy end groups allow for covalent linkage using established chemistry, and biotin end group can be used to bind streptavidin or streptavidin-linked molecules with high affinity. We have also prepared PEG lipid-CPNs using other conjugated polymers, including PFO, PFPV, and MEH-PPV. These additional NPs behave similarly to PEG lipid-PFBT nanoparticles,
with variation in size and spectral properties that most likely reflect the differences between their respective conjugated polymer starting materials.

Table 1 TEM size of PFBT nanoparticles prepared with different varieties of PEG lipids

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Diameter (mean ± FWHM)/nm</th>
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<tbody>
<tr>
<td>Methoxy 550 Mr PEG lipid-PFBT</td>
<td>26±5</td>
</tr>
<tr>
<td>Methoxy 1000 Mr PEG lipid-PFBT</td>
<td>26±4</td>
</tr>
<tr>
<td>Methoxy 2000 Mr PEG lipid-PFBT</td>
<td>24±5</td>
</tr>
<tr>
<td>Biotin 2000 Mr PEG lipid-PFBT</td>
<td>21±5</td>
</tr>
<tr>
<td>Carboxy 2000 Mr PEG lipid-PFBT</td>
<td>24±5</td>
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The size of PFBT nanoparticles formed in the presence of PEG lipid was characterized by both TEM and DLS. Representative TEM data and size distribution are shown in Fig. 1; TEM diameter obtained for PEG lipid- PFBT nanoparticles with different functional end group are listed in Table 1. Mean PEG lipid-PFBT particle size is ca. 24 nm, and is insensitive to changes in PEG lipid end groups and PEG Mr tested. DLS diameters for PEG lipid-PFBT NPs are 20-30 nm larger than those measured by TEM. Unlike TEM measurements, DLS size measurements reflect the hydrodynamic diameter and are expected to be somewhat higher than those measured by TEM, particularly for
PEG coated nanoparticles, since extension of long PEG groups into solution will be accompanied by significant solvation not present under TEM conditions. Differences between TEM and DLS measurements observed here are similar to those reported for PEG coated semiconducting polymer nanospheres [22]. However, absolute size values obtained by DLS measurements are accurate only for monodisperse particles; 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 in terms of absolute size.

Fig.1 Representative TEM images and size distribution for PEG lipid-PFBT nanoparticles. (A) TEM image of methoxy 2000 Mₐ PEG lipid-PFBT nanoparticles. Images are acquired at 120 kV on a cryostage at liquid nitrogen temperature and are shown at 120,000 magnifications. Scale bar is 500 nm. (B) Histogram of size distribution and fit to a Gaussian function for the methoxy 2000 Mₐ PEG lipid-PFBT nanoparticles showing the average diameter to be 24±5 nm (mean±FWHM).
Using DLS, we also measured the \( \zeta \) potential of the PEG lipid-PFBT NPs. These data provide initial evidence for PEG lipid incorporation into these CPNs. Since \( \zeta \) potential of PEG lipid CPNs reflects the charge of all constituent materials in NP, incorporation of different functionalized PEG lipid into the CPN will result in \( \zeta \) potentials that reflect the charge of the different end groups, in addition to the phospholipid and constituent conjugated polymer. PFBT nanoparticles prepared with carboxy PEG lipid have negative \( \zeta \) potential (-38±1 mV), reflecting the negative charge on charged end group as well as the phospholipid, while biotin and methoxy PEG lipid-CPNs have smaller negative \( \zeta \) potentials (-9±1 mV and -6±1 mV, respectively) that reflect the neutral end group as well as the negative charge on the phospholipid.

Additional evidence for incorporation of PEG lipid into these CPNs comes from their observed properties, which are different than those of the corresponding unmodified CPNs. For example, 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. Similarly, 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, consistent with no significant binding to the hydrophobic filter. In contrast, CPNs prepared without PEG lipid and diluted in buffer bind to the filter in small but visible quantities in our hands, either as a result of their higher hydrophobicity, possible instability in the presence of buffer salts, or the presence
of aggregates. Most notably, we observe that PEG lipid-PFBT CPNs have higher quantum yield than the corresponding unmodified CPNs (Table 2). For example, methoxy 550 Mr PEG lipid-PFBT nanoparticles have a quantum yield of $19 \pm 1\%$, compared to the value of $12 \pm 1\%$ we measure 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. Together, these observations are consistent with incorporation of PEG lipid into the CPNs, with resulting increases in hydrophilicity and fluorescent brightness.

**Table 2. Quantum yields for PFBT CPNs (fluorescein in 0.1 M NaOH as reference)**

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Quantum yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methoxy 550 $M_r$ PEG lipid-PFBT</td>
<td>19±1%</td>
</tr>
<tr>
<td>Biotin 2000 $M_r$ PEG lipid-PFBT</td>
<td>17±1%</td>
</tr>
<tr>
<td>Carboxy 2000 $M_r$ PEG lipid-PFBT</td>
<td>18±1%</td>
</tr>
<tr>
<td>PFBT (unmodified)</td>
<td>12±1%</td>
</tr>
</tbody>
</table>

We hypothesize that PEG lipid-CPNs form *via* a process analogous to that proposed for unmodified CPNs formed *via* reprecipitation. When the conjugation polymer is diluted, nano-particles experience a sudden change in the microenvironment of solvent, leading to collapse of the hydrophobic 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 and are incorporated into the nanoparticles during chain collapse; the bidentate lipid tail inserts into the CPN core and is retained there by hydrophobic interactions, while the hydrophilic PEG group protrudes out into the aqueous solution. Hence, the CPN surface is modified with hydrophilic functionalized PEG that helps prevent aggregation, improves biocompatibility, and provides end groups that can be used for conjugation and labeling. A similar structure has been proposed for polymer-encapsulated quantum dot nanoparticles coated with PEG lipid [17, 18].

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 relative to unmodified particles. It is known that conjugated polymer fluorescence is quenched by interactions between polymer fluorophores [23]. For example, polymer aggregation lowers the quantum yields of conjugated polymer in aqueous or hydrophilic solutions. Similarly, unmodified CPNs have substantially lower quantum yields than their constituent conjugated polymer precursors, presumably due to interactions between polymer segments after chain collapse [24]. As a result, 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 that contributes 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 spectrum [25]. Indeed a comparison of high resolution absorption spectra of methoxy 2000 Mr 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.

We cannot rule out the possibility that PEG lipid-CPNs prepared here form by micelle entrapment of conjugated polymer nanoparticles, as proposed for PEG-capped polymer coated QDs [20] and semiconducting polymer nanospheres [21]. A study of micelle formation for 2000 Mr PEG lipid reports a critical micellar concentration (CMC) value that is approximately micromolar, and a measured micelle size of ca. 17 nm [26]. The PEG lipid concentrations used in our experiments (17-83 µM) are therefore above the CMC, and micelles may be present in solution prior to addition of conjugated polymer. However, the thermodynamic stability of PEG lipid micelles of such small size is predicted to be low [27, 28], and our CPNs are not prepared under conditions that favor micelle formation. We carried out control experiments to investigate the presence of lipid micelles in our nanoparticle preparations; no measurable light scattering was observed in PEG lipid solutions at concentrations up to 83 µM (data not shown), indicating that micelle, if present, were not observable. Since the reported PEG lipid micelle size is comparable to or smaller than the reported diameter for unmodified CPNs (e.g. 10 to 30 nm for PFBT [12]), it is unlikely that partitioning of independently precipitated CPNs
into preformed PEG lipid micelles could occur. If CPNs present inside are 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 and blue shift in absorbance maximum. In this case, the final PEG lipid-CPN structure would be indistinguishable from that resulting from the proposed coprecipitation mechanism.

2.3.2 OPTIMIZATION OF PEG LIPID-CPN PREPARATION

To determine preparation conditions that result in maximal incorporation of CPNs with PEG lipid, experiments were carried out that altered the concentration of PEG lipid in solution. Initial nanoparticles preparations used 50 µg ml⁻¹ PEG lipids. However, PEG lipid-CPNs were also prepared using 25 µg ml⁻¹, 100 µg ml⁻¹, 150 µg ml⁻¹, and 200 µg ml⁻¹ 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 µg ml⁻¹ PEG lipid concentration. When nanoparticles were prepared in reduced concentrations of PEG lipid (less than 20 µg ml⁻¹), a portion of the nanoparticle preparation bound to the membrane filter, reflecting increased hydrophobicity that presumably results from inadequate incorporation of PEG lipid into CPNs. These analyses suggest that maximum incorporation of the PEG lipids tested here is achieved for preparations that use a 50 µg ml⁻¹ PEG lipid solution, and increased PEG lipid concentrations have no effect.
Table 3: Apparent hydrodynamic (DLS) size for carboxy 2000 $M_r$ PEG lipid- PFBT CPNs prepared with a range of starting PEG lipid concentrations.

<table>
<thead>
<tr>
<th>PEG lipid</th>
<th>Diameter (DLS)/nm</th>
<th>Polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 $\mu$g ml$^{-1}$</td>
<td>52</td>
<td>0.25</td>
</tr>
<tr>
<td>50 $\mu$g ml$^{-1}$</td>
<td>54</td>
<td>0.22</td>
</tr>
<tr>
<td>100 $\mu$g ml$^{-1}$</td>
<td>52</td>
<td>0.23</td>
</tr>
<tr>
<td>150 $\mu$g ml$^{-1}$</td>
<td>58</td>
<td>0.22</td>
</tr>
<tr>
<td>200 $\mu$g ml$^{-1}$</td>
<td>56</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Table 4: Apparent hydrodynamic sizes for carboxy 2000 $M_r$ PEG lipid-PFBT CPNs prepared from a range of starting conjugated polymer concentrations.

<table>
<thead>
<tr>
<th>[PFBT]$_{ini}$ factor</th>
<th>[PFBT]$_{fin}$ (DLS)/nm</th>
<th>Dilution Index (PDI)</th>
<th>Diameter (DLS)/nm</th>
<th>Polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 ppm</td>
<td>1 ppm</td>
<td>1000</td>
<td>86</td>
<td>0.35</td>
</tr>
<tr>
<td>500 ppm</td>
<td>50 ppm</td>
<td>10</td>
<td>62</td>
<td>0.15</td>
</tr>
<tr>
<td>250 ppm</td>
<td>25 ppm</td>
<td>10</td>
<td>59</td>
<td>0.15</td>
</tr>
<tr>
<td>200 ppm</td>
<td>20 ppm</td>
<td>10</td>
<td>59</td>
<td>0.17</td>
</tr>
<tr>
<td>150 ppm</td>
<td>15 ppm</td>
<td>10</td>
<td>58</td>
<td>0.19</td>
</tr>
<tr>
<td>100 ppm</td>
<td>10 ppm</td>
<td>10</td>
<td>58</td>
<td>0.19</td>
</tr>
<tr>
<td>50 ppm</td>
<td>5 ppm</td>
<td>10</td>
<td>58</td>
<td>0.19</td>
</tr>
<tr>
<td>10 ppm</td>
<td>1 ppm</td>
<td>10</td>
<td>58</td>
<td>0.22</td>
</tr>
</tbody>
</table>
To see the dependence of PEG lipid-CPN size on initial polymer concentration, PEG lipid-PBFT 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. The size of the resulting nanoparticles was evaluated by DLS. At these starting concentrations of conjugated polymer (10 to 250 ppm), the apartment hydrodynamic diameter of the PEG lipid-PFBT nanoparticles is independent of the starting concentration, and the only outcome of increased starting polymer concentration is increased nanoparticle concentration. However, at higher polymer concentrations, apparent particle size increases. Ten-fold dilutions of polymer concentrations above 500 ppm resulted in larger observed particle size by DLS. It has been observed that the size of unmodified CPNs also varies with starting polymer concentration [12], although no systematic study of this dependence has been reported.

2.3.3 FLUORESCENCE PROPERTIES OF PEG LIPID-CPNs

We evaluated the spectroscopic properties of PEG lipid-CPNs. Notably, the absorption and emission maxima of PEG lipid-CPNs (Fig. S1) are close to those of particles prepared in the absence of PEG lipid for PFBT, PFO, PFPV, and MEH-PPV PEG lipid nanoparticles [12, 24, 29]. 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 [24, 29]. 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. Similar to unmodified CPNs, PEG lipid-CPNs show good photostability (Fig. S2). PEG lipid-PFBT nanoparticles show the best photostability in our experiments of unmodified CPNs [12]. Together; these data indicate that apart from increases in quantum yield, incorporation of PEG lipid does not substantially alter the spectroscopic behavior of CPNs.

**Fig: S1 Absorbance and fluorescence spectra of PEG-Lipid CPNs.** (A) Normalized absorption spectra for biotin PEG lipid PFO (solid line), PFPV (dashed line), PFBT (dotted dashed line), and MEH-PPV (dotted line) nanoparticles; (B) Normalized fluorescence emission spectra for biotin PEG lipid PFO (solid line), PFPV (dashed line), PFBT (dotted dashed line), and MEH-PPV (dotted line) nanoparticles. Fluorescence emission spectra were acquired using excitation maxima ($\lambda_{\text{ex}}$(PFO) = 384 nm, $\lambda_{\text{ex}}$(PFPV) = 458 nm, $\lambda_{\text{ex}}$(PFBT) = 460 nm, $\lambda_{\text{ex}}$(MEH-PPV) = 498 nm).
Fig: S2 Photostability of biotin PEG-lipid CPNs. Biotin 2000 Mr PEG-Lipid CPNs were continuously illuminated with 250µW excitation light source ($\lambda_{ex}$(PFO) = 384 nm; $\lambda_{ex}$(PFPV) = 458 nm $\lambda_{ex}$(PFBT) = 460 nm $\lambda_{ex}$(MEH-PPV) = 498 nm) for 2 hours. Fluorescence emission was monitored ($\lambda_{em}$ (PFO) =435 nm; $\lambda_{em}$(PFPV) =518 nm; $\lambda_{em}$(PFBT) = 535 nm; $\lambda_{em}$(MEH-PPV) =590 nm. Spectra correspond for PFBT, PFO, MEH-PPV and PFPV from top to bottom.

2.3.4 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, and minimization of aggregation is desirable to increase nanoparticles shelf life. Since incorporation of a PEG lipid into CPNs result 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 DSL at intervals over 60 days (Fig. 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 solutions. 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.

Fig. 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 enclosed within the data symbol. Note that x-axis is not linear.

2.3.5 STABILITY OF PEG LIPID-CPNs TO CONCENTRATION

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 being concentrated by ultrafiltration with a centrifugal concentrator. In these experiments, a 28 ppm solution of carboxy PEG lipid-PFBT nanoparticles solutions was concentrated to a final concentration of 625 ppm. Portions of this concentrated solution were rediluted 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 ultrafiltration membranes, and are currently concentrated by dilution in glycerol followed by vacuum evaporation [30] to yield CPN solutions of nanoparticles in glycerol, with an upper concentration limit of about 200 ppm. Solutions concentrated via this method are not useful for cell studies, as glycerol is a potent osmolyte. The amenability of PEG lipid CPNs to concentration via ultrafiltration will allow their more widespread use in biological system that require increased CPN concentration, or that require concentrations not achievable for unmodified CPNs.
2.3.6 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 incorporated into these CPNs 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 PFBT PEG lipid-CPNs with biotin end groups were used to bind streptavidin. 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, bound nanoparticles were removed by competition with free biotin and the magnetic beads removed with a strong permanent magnet. As shown in Fig.3, the resulting supernatant 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 (Fig.3, dashed line), which lack the biotin functionality necessary for binding to the beads. These results demonstrate both successful incorporation of biotin PEG lipid end group 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, rinsed to remove non-binding particles, and then air-dried. As shown in the Fig. 4, significant numbers of near diffraction-limited spots of nanoparticle fluorescence can be observed (Fig.4A),
indicating biotin-functionalized PEG lipid CPNs binding to streptavidin on the glass surface. Spots with a range of brightness are observed, with brighter spots probably reflecting multiple nanoparticles bound to streptavidin clusters and/or nanoparticles clusters formed during drying. In contrast, very little nanoparticle fluorescence is observed in the control plates incubated with PEG lipid-CPNs with carboxy end groups (Fig.4 C). Together, these data indicate binding of biotin PEG lipid-CPNs to the streptavidin coated glass.

Fig 3: Fluorescence emission spectra of biotinylated PEG lipid PFBT nanoparticle pull down 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 supernatant was recorded (solid line; \( \lambda_{ex} = 460 \) 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).
Fig. 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 ($\lambda_{ex} = 495$ nm; $\lambda_{em} = 510$ 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.
Surprisingly, careful examination of the CPN signal observed in these cover glass 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 a distance of less than 225 nm apart. However, variations in the intensity of individual sites of PFBT fluorescence can be used to indicate varying numbers of CPNs in individual 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 was 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 the Fig. 4B, the intensity distribution of the near diffraction-limited regions shows a narrow distribution of biotin PEG lipid-CPN spots of approximately constant intensity (Fig. 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 for these 2000 M_r PEG lipid-PFBT Nps (Fig.1), the observed size stability of these nanoparticles in solutions over time (Fig.2) and the precedent for nonaggregation of PEG coated particles in previously published systems [19, 31], 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 particles 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.
2.3.7 TARGETING OF BIOCONJUGATED PEG LIPID CPN TO CD/32 RECEPTORS ON CELL SURFACE

The properties of PEG lipid-CPNs such as their high extinction coefficient, bright fluorescence, photostability, and functionalization indicate 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 these experiments, 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 in a sandwich format.

The result was Ab-conjugated nanoparticles that specifically labeled antibody-tagged receptors on the cell surface. Fig.5 shows the differential interference contrast and fluorescence images taken of labeled cells. Localized nanoparticle fluorescence is observed on the periphery of the cell, as is typical for membrane localization. Control experiments performed either without streptavidin or using carboxy modified nanoparticles instead of biotinylated nanoparticles showed no fluorescence (data not shown), indicating that observed binding of biotinylated PEG lipid-PFBT Nps does not represent nonspecific adsorption to the cell membrane. Together, these data demonstrate that PEG lipid-CPNs can target specific tagged proteins on the cell surface. To our knowledge, these results represent the first report of targeted delivery of CPNs to individual sites on the cell surface.
Fig.5 Biotinylated PEG lipid-PFBT nanoparticles targeted to cell surface receptors. (A) Differential Interference Image (DIC) of fixed J774 A.1 cells; (B) fluorescence image of fixed J774 A.1 cells labeled with biotinylated PFBT nanoparticles. Scale bar is 25µm. J774 A1 macrophage cells which express CD 16/32 (F\(_c\) receptor) were paraformaldehyde fixed and incubated with biotinylated anti-CD 16/32 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.

2.4 CONCLUSION

Incorporating functionalized PEG lipids into CPN is a simple method for preparing extremely bright biocompatible nanoparticles with enhances 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 demonstrate 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.

2.5 REFERENCES


CHAPTER THREE

THE RELATIVE BRIGHTNESS OF PEG LIPID-CONJUGATED POLYMER NANOPARTICLES AS FLUID PHASE MARKERS IN LIVE CELLS

3.1 INTRODUCTION

Analyses of cellular functions routinely employ fluorescence-based techniques such as fluorescence microscopy and flow cytometry. For example, confocal and wide field imaging techniques are used to visualize cell and organelle structure, while flow cytometry takes advantage of fluorescently labeled cells to analyze, sort, and classify populations of cells for a variety of applications. However, the success of both fluorescence imaging and flow cytometry depends on the availability of bright photostable fluorescent probes. Small molecule dye labels such as fluorescein or Texas red have been widely used, particularly as labels for endocytic compartments in live cells [1]. However, these organic fluorophores tend to photo-bleach rapidly [2] and are removed from the cellular environment via efflux pathways such as organic anion transporters [3] unless tethered to larger polymer molecules such as dextrans. Further, these fluorophores do not have sufficient fluorescent signal for straightforward single molecule imaging [4].

Fluorescent nanoparticles are a better photon source for biological applications due to their improved brightness, photo-stability, and lower susceptibility to transport out of the cell compared to small molecule labels [2, 5-7]. In addition, the nanoparticle surface can be coated with specific external shell materials like polyethylene glycol (PEG) lipid[8-11], amphiphilic block copolymer [9], or silica [12] to increase
nanoparticle solubility and stability in aqueous environments and provide functional
groups for targeting to cell surface specific receptors and active delivery to specific
cellular locations [11, 13, 14]. However, some nanoparticles result in cellular toxicity,
which can arise from either core nanoparticle or the external shell composition [15-17].
Cytotoxicity is most noted for semiconductor quantum dots, due to the possibility of
leaching heavy metal ions such as highly toxic Cd\textsuperscript{2+} from the nanoparticle core.

Highly fluorescent organic dye polymers, such as PFPV (poly[\{9,9-dioctyl-2,7-
divinylenefluorenylene\}-alt-co-\{2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene\}], MEH-
PPV(poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene], PDHF (poly(9,9-
dihexylfluorenlyl-2,7-diyl) and PFBT (poly[(9,9-dioctyfluorenyl-2,7-diyl)-co-(1,4-benzo-
\{2,1,3\}-thiadazole)]) have been used to synthesize extremely bright conjugated polymer
nanoparticles by a simple reprecipitation method [11, 18]. According to this method,
when rapidly diluted from an organic solvent phase into aqueous solution, hydrophobic
polymer molecules fold to exclude water from their surfaces, creating nanoparticles with
concentrated intrinsic fluorescence, and extremely high absorption cross sections \textit{in vitro}.
For example, unmodified PFBT nanoparticles have a reported extinction coefficient of 5
\times 10^7 \text{ M}^{-1}\text{cm}^{-1}, a value \textit{ca.} 100-fold greater than quantum dot nanoparticles of similar size
and 1000-fold greater than typical small molecule dyes [12]. In addition, nanoparticle
excitation and emission can be tailored by mixing two different polymers, or doping with
specific dyes [19, 20]. These physical and photophysical properties make reprecipitated
conjugated polymer nanoparticles ideal tools for imaging in biological systems, including
single particle tracking [21], multicolor applications [22] and biological sensor
development [23, 24]. Conjugated polymer nanoparticles can also be prepared via miniemulsion [25-27] although with somewhat lower yield and typically much larger observed nanoparticle diameters. As a part of efforts to tether conjugated polymer nanoparticles to recognition molecules for cellular targeting, we have prepared reprecipitated conjugated polymer nanoparticles in the presence of amphiphilic functionalized PEG lipid, to create conjugated polymer nanoparticles encapsulated with functionalized PEG [11]. These new functionalized PEG lipid-coated conjugated polymer nanoparticles possess improved properties relative to uncoated conjugated polymer nanoparticles, including resistance to aggregation, greater solubility in aqueous solution, and increased quantum yield [11]. It has been demonstrated that uncoated CPNs contain potentially non-reproducible surface chemical defects resulting from surface polymer oxidation that occurs during preparation [28], and surface coating by PEG may ameliorate this effect.

Because of the extremely high fluorescent brightness of conjugated polymer nanoparticles in vitro, it has been suggested that these nanoparticles are attractive candidates for use as cellular labels in biological imaging. Indeed, a series of manuscripts have shown that conjugated polymer nanoparticles are efficiently taken up into cells [29-34]. We have reported that uncoated conjugated polymer nanoparticles can be successfully used as endocytic markers [29]. However, figures of merit typically used to describe conjugated polymer nanoparticles’ brightness, including extinction coefficient, quantum yield, and fluorescence cross-sectional area, are obtained outside the cell, and do not necessarily translate directly into brightness inside the cell. For example,
fluorophore signal may change or show diminished brightness inside cells, as has been reported for several organic dyes [35, 36]. In addition, fluorophores may be taken up into cells with a range of uptake efficiencies, leading to variations in relative intracellular signal(s) that reflect differences in intracellular concentration rather than the brightness of individual fluorophores. As a result, direct intracellular comparison of different fluorophores under biological conditions is required to appropriately assess relative brightness for biological imaging. In this manuscript, we compare PEG lipid-coated conjugated polymer nanoparticles to commercially available Qdots and small molecule organic dyes with respect to spectral properties, mode and rate of cellular uptake, final destination in the macrophage cell line J774A.1, and relative brightness inside the cell. The resulting data indicates that these PEG lipid-coated conjugated polymer nanoparticles are exceptional candidates for biological labeling applications, including both cellular imaging and flow cytometry.

3.2 MATERIALS AND METHODS

3.2.1 REAGENTS

The conjugated polymer PFBT (Mr = 48,000; polydispersity = 2.7) was purchased from American Dye source (Quebec, Canada). Methoxy 2000 Mr polyethylene glycol lipid was purchased from Avanti Polar Lipids. The J774A.1 mouse macrophage cell line was purchased from American Type Culture Collections. Texas red dextran (TR-dex) (Mr = 10,000), AF488-dex (Mr = 10,000), and carboxy Qdots 525 were purchased from Invitrogen. Allophycocyanin (APC) labeled lysosome-Associated Membrane Protein 1
(LAMP-1) monoclonal antibody was purchased from Southern Biotech. All other chemicals used were purchased from Sigma-Aldrich, Fisher Scientific, or VWR.

3.2.2 PREPARATION AND CHARACTERIZATION OF CPNs

The method of preparation of PEG lipid-PFBT nanoparticles is described in detail elsewhere [11]. Briefly, one ml of PFBT (250 ppm) dissolved in HPLC grade tetrahydrofuran (THF) was diluted rapidly into 9 ml distilled-deionized H₂O containing 50 ppm methoxy-capped 2000 Mₗ PEG lipid under mild sonication to facilitate fast mixing. THF was removed from the suspension under vacuum evaporation, and filtered through a 220 nm PVDF syringe filter. A very dilute solution (ca. 100 pM) of the resulting nanoparticles was spread on formvar copper grids by drop casting. The size of the nanoparticles was measured using a Hitachi H7600 transmission electron microscope (TEM) at 120 kV on a cryostage cooled with liquid nitrogen. The diameter of the CPNs was measured with Image J. The determined particle diameters were fit in to a Gaussian distribution using Sigma Plot (Systat). Hydrodynamic size of the nanoparticles was measured by dynamic light scattering (DLS) using a Malvern Zetasizer (ZS90) at 25°C as previously described [11]. Nanoparticle concentration was estimated from the mass of conjugated polymer diluted into aqueous solution and the TEM size, assuming complete polymer to nanoparticle conversion, as previously described [29].
3.2.3 FLUORESCENT INTENSITY MEASUREMENTS OF FLUOROPHORES (AF488-DEX, QDots, AND CPNs)

Fluorescence emission spectra of AF488-dex, Qdots, and CPNs were acquired using a photon counting spectrofluorometer (Photon Technology International; QM-4). In order to achieve measurable fluorescence intensity, a 500-µM stock solution of AF488-dex was diluted to 112 nM; an 8 µM Qdot stock was diluted to 22.4 nM, and 6 nM CPN stock was diluted to 0.6 nM in water. The emission spectra of the diluted solutions were measured from 495 nm to 650 nm using 488 nm excitation, (4 nm bandpass) for all of the three fluorophores. Fluorescence emission of each of the fluorophores was also recorded under similar conditions but using the corresponding excitation maximum for each fluorophore (494 nm for AF488-dex, 400 nm for Qdots and 460 nm for CPNs).

3.2.4 CELL CULTURE

Mouse macrophage-like J774A.1 cells were grown in Dulbecco’s Modified Eagles Medium (DMEM; Mediatech) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS; Hyclone), L-glutamine (2 mM), penicillin (100 units/mL) and streptomycin (100 µg/mL), in a humidified incubator with 5% CO₂ at 37°C. The viability of the cells was 97% or more with each passage, as determined by Trypan blue exclusion assay.
3.2.5 FLUORESCENCE MICROSCOPY OF FLUOROPHORE UPTAKE

J774A.1 cells were grown in optical bottom culture plates until about 70% confluent, at which time the cells were incubated with either 0.6 nM (2.7 ppm) CPNs, 22.4 nM Qdots, and 112 nM AF488-dex overnight (16 h) in DMEM + 10% FBS at 37°C and 5% CO2. For co-localization experiments, 200 nM TR-dex was also added together with each fluorophore. Following incubation, cells were washed three times with Ringer’s Buffer (RB; 10 mM HEPES, 155 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 2 mM NaH2PO4, 10 mM glucose, pH 7.2-7.4), and incubated without extracellular fluorophore under culture conditions (chased) for ≥4 hours prior to imaging. Under these chase conditions, sufficient time has elapsed that all endocytic cargo is assumed to be delivered to terminal vacuoles [1], which for fluid phase uptake in macrophage cells is the lysosome [37],[1]. Fluorescence imaging was performed on an inverted epifluorescence microscope (Olympus IX71) with xenon arc lamp excitation and a 60X/1.45NA objective. CPNs, Qdots and AF488-dex were viewed with 494 nm excitation (20 nm bandpass) and 531 nm emission (22 nm bandpass) while TR-dex was viewed with excitation wavelength 575 nm (25 nm bandpass) and emission at 624 nm (40 nm bandpass).

3.2.6 FLOW CYTOMETRIC STUDIES OF FLUOROPHORE UPTAKE

J774A.1 cells grown in 35 mm culture dishes were incubated with Qdots, AF488-dex, and CPNs separately in DMEM containing 10% heat inactivated FBS. For time
course studies, cells were incubated with fluorophore for each of 1, 2, 4 and 8 h, with 0.6 nM (2.7 ppm) CPNs, and with Qdots (22.4 nM) and AF488-dex (112 nM) concentrations that were 37-fold and 186-fold higher, respectively. At individual time points, cell plates were chilled on ice and the cells were detached from the surface by pipetting. Detached cells were pelleted, and washed 3X with RB before suspension in RB. Fluorescence from 10,000 cells was measured by flow cytometer (BD FACScan) at an excitation wavelength of 488 nm with the emission in the green channel. The data from FACScan were analyzed using FlowJo software (Treestar). In the dose dependent studies, cells were incubated with each fluorophore for 8 hours over the concentration range(s) of 22 to 600 pM (CPNs), 1.4 to 22.4 nM (Qdots), or 4.5 to 112 nM (AF488-dex). Cells were prepared for flow cytometer analysis as for the time course studies.

3.2.7 IMMUNOCYTOCHEMICAL ANALYSIS

J774A.1 cells grown in glass bottom culture dishes were incubated with fluorophores at 37°C for 16 hours in a 5% CO₂ incubator. Cells were washed 3x with RB, and then chased for 4 hours in DMEM culture medium. The cells were fixed with 4% paraformaldehyde in RB at 37°C for 10 minutes, and washed 3x again with RB before blocking with blocking buffer (RB containing 1.5% BSA, 0.3% Triton-X solution) for 2 hours at 4°C. The cells were next incubated with the LAMP-1 specific antibody labeled with Allophycocyanin (APC) (200-fold dilution of 0.1 mg/ml stock solution) for 16 hours in RB containing 1.0% BSA and 0.3 % Triton-X at 4°C. The cells were washed with
blocking buffer 3x 15 minutes each at room temperature. Fluorescence imaging was performed by inverted epifluorescence microscope (Olympus IX71) with xenon arc lamp as excitation source and a 60X/1.45NA objective. The fluorescence of each of CPNs, Qdots, and AF488-dex were observed with 494 nm excitation (20 nm bandpass) and emission of 531 nm (22 nm bandpass); Allophycocyanin fluorescence of the antibody was observed with 575 nm excitation (25 nm bandpass) and emission of 624 nm (40 nm bandpass).

3.2.8 BLOCKING NANOPARTICLE UPTAKE USING INHIBITORS OF MACROPINOCYTOSIS

Known inhibitors of macropinocytosis were used to help elucidate the mechanism of cellular uptake of CPNs, as described in Fernando et al. [29]. Briefly, J774A.1 cells were plated in 35 mm tissue culture plates and grown to ~70% confluence. The cells were preincubated with methyl-β-cyclodextrin (2.5 mg/mL final concentration), wortmannin (100 ng/ml final concentration), and LY294002 (20 µg/ml final concentration) in culture medium for 30 min, followed by incubation of cells with 0.60 nM (2.7 ppm) CPNs for 1.5 – 2 hours, also in media. Methyl-β-cyclodextrin and wortmannin are insoluble in media and require dilution in DMSO; cells treated with these inhibitors were therefore exposed to small amounts of DMSO. An additional vehicle control was performed in which cells were exposed to equivalent amounts of DMSO in the absence of inhibitors prior to incubation with CPNs. Cell processing, flow cytometry measurements and data
analysis were carried out as described above. Statistical analysis of the mean fluorescence for each treatment compared to the untreated and vehicle controls was done using ANOVA in SigmaPlot. We have previously demonstrated that under these conditions, there is less than 10% cytotoxicity from the inhibitor alone [29], indicating that observed decreases in CPN uptake in the presence of individual inhibitors do not reflect inhibitor cytotoxicity.

3.2.9 CYTOTOXICITY STUDIES

The Cell Titer Blue assay was used to assess cytotoxicity of CPNs. J774A.1 cells were plated at 10K/well in a black 96-well plates in DMEM + 10% FBS at 37°C and 5% CO₂ and incubated with CPNs in culture medium for 16-18 hours at concentrations ranging from 0 ppm (control) to 67.5 ppm (15 nM). The background fluorescence from the CPNs at λ_{ex} = 546 and λ_{em} = 585 nm was recorded using a Genios top reading fluorescence plate reader (Tecan). Cell Titer Blue reagent was then added to all according to manufacturer’s instructions, incubated with the cells for an additional 2 hours, and then the fluorescence in each well was remeasured. Individual well background fluorescence was subtracted from the total fluorescence to determine Cell Titer Blue fluorescence and then converted to percentage viability versus the untreated control wells. Statistical analysis of cell viability versus the untreated control was done using ANOVA in SigmaPlot followed by Bonferroni comparison; P-values less than 0.05 were used to conclude a significant difference between samples and controls.
3.3 RESULTS AND DISCUSSION

In this study, we explore the suitability of methoxy-functionalized PEG lipid-coated conjugated polymer nanoparticles for application as intracellular probes for cellular imaging and flow cytometry. We compare the advantages these nanoparticles offer over commercially available fluorophores such as Qdots and Alexa fluor dextran for ex vivo cell labeling in J774A.1 cells. Our goal was to determine the relative brightness of the CPNs to Qdots and organic dyes when loaded into cells, their respective uptake efficiency and mechanism of cell entry, and their final intracellular localization. We also evaluate the cytotoxicity of PEG lipid-CPNs.

In these studies, we use CPNs as a representative PEG lipid conjugated polymer nanoparticle. These nanoparticles were synthesized from commercially available methoxy-functionalized PEG lipid and PFBT by reprecipitation, as previously described [11]. Based on spectral behavior and functional end group reactivity, the resulting nanoparticle structure is presumed to have a fluorescent PFBT-lipid core, surrounded by a corona of PEG molecules that results in high solution stability and increased quantum yield relative to bare PFBT particles [11]. A similar behavior was observed for PEG lipid coated conjugated polymer nanoparticles prepared with other functional end groups (e.g. carboxy, biotin) that allow for conjugation to biorecognition molecules [11]. Transmission electron microscope characterization of the CPNs used here indicates that the particles are approximately spherical in shape with a mean particle diameter of 24±5 nm, as shown in Figure 1a and 1b. Dynamic light scattering (DLS) analysis of this preparation gave a DLS diameter of 59 ± 2 nm, with a moderate polydispersity index of
0.14 ±0.03. The observed size difference between TEM and DLS measurements is consistent with 20 to 30 nm differences previously reported for PEG-coated conjugated polymer nanoparticles prepared by miniemulsion [34] and presumably reflects substantial hydration of the PEG surface. Some additional inflation of measured hydrodynamic radius by small amounts of high molecular weight particles is possible in this moderately polydisperse sample, although we have seen no evidence of the existence of aggregates. The measured size for this preparation is identical to our previous preparation [11], indicating that this method produces nanoparticles of highly reproducible size. The choice of methoxy as functional end group is arbitrary here, as we have seen no impact of the functional group in PEG lipid molecule on nanoparticle spectral behavior [11] or cellular uptake.

Fig. 1 TEM characterization and size distribution of CPNs. (a) Typical TEM image of CPNs. (b) Histogram of measured CPN diameter from TEM image analysis using Image J software. Histogram fit to Gaussian. Mean diameter = 24 ± 5 nm. Scale bar = 500 nm.
Nanoparticle uptake can change as a function of particle size and surface characteristics [15, 38-41], as alteration of nanoparticle surface or size can lead to differential interaction with cell surface receptors that can facilitate uptake. Hence, direct comparisons of nanoparticle uptake are only relevant for nanoparticles with similar size and surface characteristics. To directly compare the behavior and uptake of these CPNs, we chose a representative commercially available Qdot with an amphiphilic coating and approximately equivalent size. In this case, the semiconductor CdSe or CdTe core with a Zn sulfide shell was coated with a carboxy-functionalized (proprietary) amphiphilic polymer layer. The result was a ca. 20 nm quantum dot with a hydrophilic surface. A range of commercially available fluorescent dyes are available for comparison to the CPNs; we chose to use AF488-dex, since it is among the brightest of the available organic dyes at the wavelength typically used for biological imaging and flow cytometry. As a result, signal performance of AF488-dex relative to CPNs represents a “best case” behavior for organic dyes.
Biological imaging and analysis is typically carried out with 488 nm excitation, using a 488 nm argon-ion laser. In particular, commercially available flow cytometry instruments widely used to detect cell labeling are equipped with 488 nm excitation. We compared the spectral behavior of CPNs to Qdots and AF488 under these conditions. Figure 2A shows a comparison of the fluorescence intensities of the three fluorophores at an excitation wavelength of 488 nm. In this case, CPNs are so much brighter than either Qdots or organic dyes that they cannot be compared at equivalent concentrations; concentrations that allow adequate AF488 signal result in overloaded detection for CPNs. Spectra shown in Figure 2 were obtained with 0.6 nM (2.8 ppm) CPNs, while Qdots and AF488-dex concentrations were 37 and 186 fold higher, respectively. The spectra
demonstrate that CPNs have a wide emission spectrum ($\lambda_{\text{max}} = 540$ nm) compared to Qdots and AF488-dex. Based on the integrated fluorescence intensity under the spectra, we estimate that the measured signal intensity for CPNs would be 160 times brighter than Qdots and 600 times brighter than AF488 under these conditions when corrected for the differences in concentration of the different fluorophores. We note that these measurements were not obtained at the excitation maximum for either CPNs $\lambda_{\text{max}} = 460$ nm or Qdots ($\lambda_{\text{max}} = 400$ nM). When the fluorescence intensity was compared using the respective absorption maxima of each fluorophore (Fig. 2B), CPNs have still higher relative signal; CPNs are ca. 37-fold brighter than Qdots and 510-fold brighter than AF488-dextran under ideal excitation conditions. We have seen no impact of functional end group on PEG lipid coated conjugated polymer nanoparticle spectra or quantum yields; hence, data obtained for CPNs also reflect that for PEG lipid-coated PFBT nanoparticles with other PEG lipid end groups [11]. We note that single molecule brightness comparisons have been made between Qdot 565, IgG-AF488 and polystyrene PEG PFBT NP prepared using an alternate protocol [42]; the relative brightness of this alternative type of PFBT CPN was reported to be somewhat lower than we observe here.

3.3.1 RELATIVE FLUOROPHORE UPTAKE AND INTRACELLULAR BRIGHTNESS

We compared the efficiency and mechanism of CPNs uptake into J774A.1 macrophage cells with that for commercial Qdots and AF488-dex dyes under equivalent conditions. Our goal was to determine the relative brightness of the CPNs versus Qdots
and organic dyes \textit{in vivo}, and to evaluate their respective mechanism of cell entry and final intracellular location(s).

First, to determine the time course of fluorophore uptake and to quantify the resulting intracellular signal, cells were incubated with each of CPNs, Qdots, and AF488-dex over time periods up to eight hours. The intracellular fluorescence of each loaded cell was determined by flow cytometry (Fig.3). As for \textit{in vitro} experiments, cells were incubated in higher concentrations of Qdots and AF488-dex in order to place the signal from these fluorophores on the same scale as the CPN signal. As shown in Fig. 3, we were able to observe good CPN signal with an hour-long incubation at a concentration of 0.6 nM (2.7 ppm) whereas 37-fold and 186-fold higher concentrations of Qdots and AF488-dex took ca. 2 and 4 hours respectively to get a discernable fluorescent intensity. This difference in time to achieve measureable intracellular fluorescence reflects differences in fluorophore brightness, and does not necessarily imply a difference in the uptake mechanism. After 8-hour incubation, the observed intracellular CPNs fluorescence was ca. 175-fold and 1400-fold greater than Qdots and AF488-dex fluorescence intensities when corrected for the differences in fluorophore concentration bathing the cell. We note that this correction for differences in extracellular fluorophore concentration assumes equivalent uptake efficiency; apparent intracellular brightness could be inflated or diminished by differences in relative uptake efficiencies for the different fluorophores. Regardless of assumptions, these observations indicate that CPNs are efficiently taken up into this cell type, and have remarkably bright intracellular fluorescence.
Fig. 3. Time course of fluorophore uptake by cells. J774A.1 cells were pulsed with AF488-dex (black; 112 nM), Qdots (light grey; 22.4 nM), or CPNs (dark grey; 0.6 nM) for the indicated times, and cell-associated fluorescence was analyzed by flow cytometry. Error bars represent the standard deviation of the mean fluorescence at 488 nm excitation for at least 3 independent measurements of 10,000 cells.

To examine the relationship between extracellular fluorophore concentration and intracellular fluorescence for CPNs, Qdots, and AF488-dex, J774A.1 cells were incubated for 8 hours with each of the three fluorophores over a range of concentrations; as for previous experiments, concentrations of Qdots and AF488-dex were 37-fold and 186-fold higher than that used for CPNs. The resulting cell-associated fluorescence under each condition was quantified by flow cytometry. As shown in Fig. 4, cellular uptake of each fluorophore resulted in dose-dependent cellular fluorescence detectable by flow cytometry. These data indicate that extremely low concentrations of the CPN label can be detected in live cells using a standard flow cytometer equipped with a <15 mW argon ion laser. Depending on the sensitivity of the individual cytometer, even lower concentrations
of CPNs could be detectable. The higher intracellular signal of CPNs compared to Qdots and AF488-dex is highlighted by their relative detection limits under these conditions. While the observed detection limit for CPNs was 19 pM (86 ppb), corresponding detection limits for Qdots and AF488-dex were 980 pM and 11.2 nM, respectively.

![Graphs](image)

**Fig.4. Dose dependent uptake of CPNs, Qdots, and AF488-dex in J774A.1 macrophage-like cells.** Individual graphs represent uptake of (a) AF488-dex; (b) Qdots; and (c) CPNs. Cells were pulsed with different concentrations of fluorophore for 8 hours, washed, and analyzed for cell-associated fluorescence by flow cytometry. Data reflects the mean fluorescence at 488 nm excitation for at least 3 independent measurements of 10,000 cells. Normalized fluorescence intensity at each dosage reflects the ratio of mean fluorescence intensity value at that dosage to mean fluorescent intensity value of the highest dosage.

CPN concentrations used in this study are low. CPNs form in response to dilution from an organic phase to an aqueous phase, resulting in a stock solution with low CPN concentrations. It should be noted that we have further diluted this low concentration into media for cell studies. However, given the extremely bright fluorescence of these particles, these very low loading concentrations are more than adequate, and we have investigated cell loading over the range of concentrations likely to be used. Also, we have previously demonstrated that the high solution stability of PEG lipid-coated conjugated
polymer nanoparticles allows these particles to be concentrated via ultra-filtration [11]. Hence, if higher concentrations were required for specific experiments, such as toxicity studies, these low concentration stock solutions could be concentrated to allow treatment of cells with dramatically more concentrated CPN solutions.

3.3.2 FLUOROPHORE UPTAKE MECHANISMS

Application of different fluorophores as labels for cellular imaging or flow cytometry requires that their final intracellular location be established. For example, dextran-coupled dyes have been widely used as intracellular labels, an application that takes advantage of their known fluid-phase uptake and trafficking to lysosomes [1]. To investigate the uptake and cellular trafficking of CPNs, and to compare it to that for Qdots and AF488-dex, a series of experiment were performed in J774A.1 macrophage cells.

In the first set of experiments, cells were incubated in fluorophore solutions overnight in the presence of TR-dex, washed to remove any extracellular fluorophore, and then incubated under culture conditions (chased) for at least an additional 4 h prior to imaging. Since dextran-labeled dyes can be present in a variety of endosome types (e.g. early endosomes, late endosomes, late-endosome-lysosomal fusion compartments, lysosomes) depending on the timescale of the experiment following initial uptake [37], a long chase was necessary to allow sufficient time for all endocytic cargo to be delivered to terminal vacuole(s), which for fluid-phase uptake in macrophage cells is the lysosome [37]. A 2 h chase is generally assumed to be sufficient for complete lysosomal delivery
and macropinosomes have been shown to deliver fluid-phase cargo to lysosomes in less than 20 minutes [37]. Hence, a 4 hours chase, as chosen here, is more than sufficient to allow all fluorophore to be delivered to lysosomes. In addition, use of a long pulse and chase helped minimize cell-to-cell variation frequently observed when using shorter experimental time scales. Fig.5 demonstrates that under these conditions, each fluorophore was taken into the cell, resulting in a vesicular staining pattern of intracellular fluorescence consistent with fluorophore localization in intracellular organelles (green), rather than dispersed uniformly throughout the cytosol. A comparison of the cellular localization of each of the fluorophores (Fig. 5; green) demonstrates that the pattern of TR-dex fluorescence (red) mirrors (i.e. merges with) that for each of CPNs, Qdots, and AF488-dex in these experiments. Dextran coupled dyes are known to be taken up by host cells via fluid phase pinocytosis (usually macropinocytosis) and are trafficked along the cell’s endocytic pathway, finally accumulating in lysosomes [1]. Hence, co-localization of TR-dex and the respective fluorophores in these experiments indicates trafficking to the lysosome, and suggests simultaneous uptake via a single endocytic mechanism. These data suggest that each of these fluorophores enter the cell via a fluid-phase endocytic mechanism, and are ultimately located in membrane-bound organelles, presumably lysosomes, located in the perinuclear region of the cells. We point out that under the conditions of these experiments, cells could not phagocytose extracellular material (cells were grown in complement-free media to preclude complement-mediated phagocytosis and nanoparticles were not opsonized, to prevent Fc-receptor mediated
phagocytosis). Hence, fluorophores entering the cell through endocytic mechanism(s) in these experiments must do so through fluid-phase endocytosis, rather than phagocytosis.

**Fig. 5. Fluorophore co-localization with TR-dex.** J774A.1 cells in individual glass bottom culture dishes were pulsed overnight with fluorophores in the presence of TR-dex and chased for ≥4 h prior to imaging. Green images reflect CPN, Qdot, and AF488-dex fluorescence; red images reflect TR-dex fluorescence; in the merged image, yellow color indicates co-localization. Because of the differences in relative brightness, the different fluorophores were loaded into cells at different concentrations (CPNs = 0.6 nM; Qdots = 22.4 nM; AF488-dex = 112 nM). Scale bars = 10 µm.

Additional support for fluid-phase endocytosis as the mechanism of fluorophore entry under these conditions was provided by uptake experiments performed on ice. In these experiments, cells were bathed in the different fluorophores and incubated on ice. At low temperatures, cells cannot take in extracellular material by energy-dependent endocytic mechanisms, and any CPN fluorescence associated with cells under these conditions must result from either CPN binding to the cell surface or via energy
independent uptake mechanisms such as diffusion through the cell membrane. After washing cells with ice-cold buffer, the relative intracellular fluorescence was evaluated by flow cytometry (Fig. 3). For each of the fluorophores, very little cell-associated fluorescence can be observed under these conditions. This observation is consistent with uptake by an energy-dependent mechanism (i.e. endocytosis), and rules out simple diffusion as the mode of fluorophore uptake since some diffusion could still occur at low temperature. In addition, the lack of interaction with the cell surface indicates that uptake does not occur through receptor-mediated endocytosis, which is initiated by interactions with receptors on the cell surface. However, while the fluorescent intensity of the CPN and AF488-dex samples incubated on ice were a very small percentage of the corresponding value at 37°C (0.2% and 2%, respectively), a somewhat higher value was observed with Qdots (13% of the value at 37°C). This observation suggests that there may be small amounts of either binding to the cell membrane or energy-independent cellular uptake, such as diffusion through cellular membranes, for these Qdots.

Flow cytometry experiments cannot distinguish between intracellular fluorescence and fluorescence resulting from fluorophore adsorption to the cell surface. Hence, the low measured CPN fluorescence observed by flow cytometry at low temperature indicates that there is very little physical adsorption (or receptor binding) of CPNs to the cell surface. A PEG coating is believed to reduce interaction with the cell surface for other nanoparticles, including Qdots [9, 43]. Consequently, the observation of little or no association of CPNs with the cells at low temperature is not unexpected. Similarly, dextran-coupled dyes do not typically interact with cell surfaces [44],
consistent with the low AF488-dex fluorescence also associated with cells at low temperature. In contrast, the Qdots fluorescence associated with cells at low temperature may reflect adsorption of the proprietary amphiphilic coating to the cell surface, as well possible energy-independent uptake mechanism(s) such as diffusion through the cell membrane or receptor-mediated endocytosis.

A subsequent set of experiments were designed to further confirm that CPNs, like other fluid-phase markers, are ultimately delivered to lysosomes. LAMP-1 or CD107a is a protein that is trafficked to the membranes of late endosomes during endosomal maturation, and is retained in the lysosomal membrane [45]. Late endosomes mature to lysosomes or fuse with and/or deliver their contents to lysosomes [37], and the presence and contents of these vacuoles are closely linked [46]. Hence, while LAMP-1 cannot be used to identify lysosomes in the absence of late endosomes, it is commonly used to identify the presence of lysosome or lysosome-like late endosomes. To demonstrate that CPNs are finally localized to lysosomes in macrophage cells, immunocytochemical analysis was carried out using a Cy5-labeled anti-LAMP-1 antibody. In these experiments, a pulse-chase experiment was performed in which cells were incubated with fluorophore to allow uptake, washed, and incubated without extracellular fluorophore for ≥ 4 hours, more than sufficient time to allow delivery of endocytic cargo to lysosomes. Cells were then fixed and stained with anti-LAMP-1 antibody. As shown in Fig.6, CPN fluorescence (green) is present in large compartmentalized LAMP-1 immunostained organelles (red) near the nucleus; the fluorophore signal co-localizes with anti-LAMP-1 antibody (merge). Similar results were obtained for Qdots, and AF488-dex. Together,
these data confirm that each fluorophore is taken up by these cells via solution phase endocytosis and is ultimately trafficked to LAMP-1-containing lysosomes.

**Fig. 6. Co-localization of fluorophores with LAMP-1.** J774A.1 cells were pulsed with CPNs (0.6 nM); Qdots (22.4 nM); or AF488-dex (112 nM), followed by a ≥4 hour chase. Cells were then paraformaldehyde fixed, detergent permeabilized, and stained with an anti-LAMP 1 allophycocyanin (APC) -conjugated antibody. Green images reflect CPN, Qdot, and AF488-dex fluorescence; red images reflect APC fluorescence; in the merged image, yellow color indicates co-localization of fluorophores and LAMP 1. Scale bars = 10 µm.

We have previously demonstrated that uptake of uncoated PFBT nanoparticles in J774A.1 macrophage cells takes place via macropinocytosis. For these bare particles, uptake was inhibited in the presence of chemical compounds that interfere with individual aspects of macropinocytosis (i.e wortmannin, cyclodextrin, and LY294002), while compounds known to inhibit other uptake mechanisms had no effect [29]. We
performed a similar analysis with these PEG lipid PFBT particles. To establish that CPNs are taken up by macroinocytosis, we evaluated the sensitivity of uptake to wortmannin and LY294002, which block the action of phosphoinositol 3-kinase (PI3K) [47]. Since PI3K is required for spontaneous cell surface ruffling that is an integral part of macroinocytosis [47], inhibition of uptake in the presence of wortmannin and LY294002 indicates that nanoparticles enter the cell through macroinocytosis. As shown in Fig. 7, both wortmannin and LY294002 result in significant inhibition of CPN uptake. An additional inhibitor, methyl-β-cyclodextrin, which inhibits cholesterol formation, also significantly reduces CPN uptake. Since cholesterol is involved in cell-surface ruffling, inhibition by cholesterol is also consistent with CPN uptake via macroinocytosis. Together, these data indicate that like uncoated CPNs, PEG-coated CPNs are taken into macrophage cells via macroinocytosis. Since macroinocytosis is a nonspecific uptake mechanism that does not require interaction with cell surface receptors [48], this characterization also indicates that interaction of CPNs with the cell surface is not required for uptake, consistent with the lack of CPN interaction with cell membrane observed at low temperature.
Fig. 7. Disruption of CPN uptake by macropinocytosis inhibitors. J774A.1 cells treated with β- methyl cyclodextrin, wortmannin, and LY 294002 were incubated with identical concentrations of CPNs, and cellular uptake was evaluated by flow cytometry. Data is presented as a percentage uptake relative to cells not treated with inhibitors; CPN uptake into cells in media in the absence of inhibitors (positive control) represents 100% uptake, and cellular fluorescence in the absence of both inhibitors and CPNs (negative control) represents 0% uptake. β- methyl cyclodextrin and LY 294002 are insoluble in media and were delivered to the cells in DMSO. The vehicle control reflects experiments in which equivalent volumes of DMSO were delivered to cells in the absence of inhibitors prior to incubation with CPNs in media. Data reflects the mean cell-associated fluorescence at 488 nm excitation for at least 3 independent measurements of 10,000 cells. Error bars are standard deviations.
3.3.3 EVALUATION OF NANOPARTICLE TOXICITY

The utility of a given probe for biological imaging or analysis is compromised if the probe causes cell death or other deleterious effects. Hence, we evaluated the cytotoxicity of CPNs in cells over the concentration range likely to be used in cell imaging. First, cells were incubated with increasing amounts of CPNs for 16-18 h and the percentage of live cells was determined using the Cell Titer Blue Assay (Fig 8).

![Bar graph showing cell viability](image)

**Fig. 8. Cytotoxicity of CPNs.** Cells were incubated with the indicated concentrations of nanoparticles for 16-18 hours, and viability analyzed using the Cell Titer Blue Assay. Percent viability is relative to cells incubated without CPNs. The standard deviation is shown based on the average of the wells used for each concentration. All nanoparticle concentrations resulted in P-values > 0.05 when compared with untreated control samples.

Notably, the percentage of live J774.A1 cells in CPN treated wells were similar to the controls (i.e. cells that had not been exposed to CPNs) at every CPN concentration.
tested (P>0.05). These data indicate that CPNs have no discernable impact on cell viability over this concentration range. In addition, CPN loaded cells were examined under DIC illumination (600 x) using an epifluorescence microscope; we observed no detached cells or membrane blebbing that would reflect cell damage, even at the highest concentrations evaluated (67.5 ppm). Hence, these data indicate that these CPNs are not toxic to the macrophage cell line at the highest tested concentration. We note that the highest concentration evaluated for toxicity is ca. 25 fold higher than the working concentration needed for cell labeling.

3.3.4 DISCUSSION

We have prepared small (24 nm) conjugated polymer nanoparticles via reprecipitation in the presence of methoxy PEG lipid. This method results in highly stable nanoparticles with extremely bright fluorescence, and different preparations consistently yield particles of equivalent size [11]. Nanoparticles of similar composition but larger size can also be prepared by miniemulsion [10, 34]. Reported outstanding figures of merit for CPNs suggest their application as labels for cellular imaging and analysis [31, 32, 42]. However, intracellular conjugated polymer nanoparticles brightness had not been evaluated with respect to competing labels. In this study, we compared the performance of CPNs with widely used commercially available fluorophores to analyze suitability for biological applications, including brightness under spectral condition likely to be used for both biological imaging and flow cytometry. These comparisons use 488- or 494-nm
excitation, rather than the relative absorption maxima of either CPNs or Qdots, since these excitation wavelengths are commonly used for flow cytometry and imaging applications using argon ion lasers. \textit{In vitro} comparison of fluorophore intensity in a steady state spectrofluorometer at an excitation wavelength of 488 nm indicates that when corrected for concentration differences; measured signal intensity for CPNs is \textit{ca.} 160 times brighter than the Qdots and 600 times brighter than AF488 at this wavelength. However, the relative brightness of CPNs is much higher inside cells. We performed experiments in which cells were loaded with fluorophore by incubation with defined concentrations of fluorophore, and the fluorescence associated with those cells was evaluated by flow cytometry. Assuming equivalent cellular uptake and after correction for differences in fluorophore concentration, we estimate that the intracellular fluorescence signal of CPNs is 175 times brighter than the corresponding Qdots signal and 1400 times brighter than AF488-dex under these conditions. We note that neither CPNs nor Qdots are excited on resonance for these experiments, leading to somewhat reduced brightness for each of these fluorophores. However, the wavelengths chosen reflect conditions typically used for biological imaging and flow cytometry, and measured brightness obtained under these conditions is the appropriate comparison for these cell-based applications.

Flow cytometry experiments, as used here to assess intracellular signal, cannot distinguish between intracellular signal and signal resulting from fluorophore adsorption to the cell surface, and our reports of relative intracellular brightness assume that fluorophore signal measured by flow cytometry reflects intracellular fluorescence. This
assumption is supported by experiments in which the different fluorophores were incubated with cells on ice, and the resulting cell-associated fluorescence was measured by flow cytometry. Very little resulting fluorescence was associated with cells under these conditions. Since low temperatures would necessarily inhibit active uptake but not interaction with the cell surface, the observed negligible fluorescence under these conditions suggests that any contribution from fluorophore interaction with the cell surface is small. We point out that Qdots show the largest amount of uptake and/or cell surface adsorption when incubated on ice and it is therefore probable that some quantity of Qdots are associated with the cell surface and/or taken into the cell by mechanism(s) not available to CPNs or dextran-coupled dyes.

Analysis of the mechanism of CPN uptake indicates that these particles are taken up by J774A.1 macrophage cells via fluid phase endocytosis. Co-localization of intracellular CPNs with TR-dex and inhibition of uptake in the presence of wortmannin, LY294002, and β methyl cyclodextrin give evidence that the mode of endocytosis is macropinocytosis, similar to our previous observations of uncoated PFBT nanoparticles uptake in macrophage cells [29]. In contrast to the majority of endocytic uptake mechanisms, macropinocytosis does not require interaction with the cell surface to initiate uptake [48]; instead, extracellular material is taken into the cell via spontaneous actin-mediated ruffles that nonspecifically enclose extracellular solution. Since it has been observed that PEG coating of nanoparticles can inhibit interaction with cell surface receptors responsible for other fluid-phase uptake mechanisms [9, 43], entry of CPNs via this nonspecific fluid phase uptake mechanism is perhaps not surprising. While
macrophage cells have particularly high levels of constitutive macropinocytosis, many cell types can take in extracellular material by this mechanism [49], and would therefore be amenable to labeling with CPNs, with the modification of somewhat longer incubation time. For example, we have observed efficient conjugated polymer nanoparticle uptake into non-macrophage Chinese hamster ovary (CHO) cells (unpublished data).

Co-localization of CPN fluorescence with TR-dex and labeled anti-LAMP-1 antibodies indicates that following macropinocytic uptake, these nanoparticles are trafficked from endosomes to lysosomes. In this respect, these nanoparticles behave similarly to the range of dextran-conjugated organic dyes such as Lucifer Yellow dextran, TR-dex, and FITC-dextran that are commonly used as fluid phase markers. Such fluorophores have been widely utilized as specific labels of endocytic compartments for analysis of cell function and endocytosis [1]. We suggest that the high brightness of these PEG-lipid CPNs, combined with their easy synthesis, resistance to aggregation, and photo-stability, makes them highly attractive substitutes for historically used fluid phase markers. For example, we have shown here that these characteristics allow them to be used as intracellular markers for flow cytometry.

We have previously demonstrated that functionalized PEG lipid-coated CPNs can be conjugated to biorecognition molecules and targeted to specific cell-surface receptors [11]. Howes et al have demonstrated coupling of larger PEG lipid coated conjugated polymer nanoparticles to BSA, as proof of principle that the PEG lipid functional group can be used to couple nanoparticles to proteins for targeted delivery [34]. Depending on the biorecognition molecule used and the choice of receptor to be targeted, such
conjugation to specific biologically relevant molecules could initiate cellular uptake via receptor-mediated endocytic mechanisms, with subsequent delivery to individual cellular locations that could include the nucleus, cytosol, or individual organelles [50, 51]. In this case, targeted delivery with minimal competition from macropinocytic delivery would be made possible simply by using a cell line with low levels of constitutive macropinocytosis. In cell line with higher rates of macropinocytosis, targeted delivery could be facilitated by experimental conditions that minimize nonspecific endocytosis; targeted CPNs could be incubated with cells on ice and washed to remove CPNs not bound to cell surface receptors prior to incubation at physiological temperatures to allow endocytic uptake.

Notably, evaluations of possible CPN cytotoxicity show no impact of nanoparticles on cell viability or structure at all tested concentrations. Similar low cytotoxicity was observed for unmodified PFBT nanoparticles [29] and other conjugated polymer nanoparticles [31], indicating that the benign behavior of these nanoparticles may reflect the characteristics of the conjugated polymer core, rather than simple shielding by PEG. While loading concentrations tested for cell damage were low, they are well above concentrations likely to be used for cell studies, given the extreme fluorescent brightness of this label.

3.4 CONCLUSION

A dogma of fluorescence labeling in cellular biology is that the amount of fluorophore probe added to the cells is sufficiently small so as to not perturb cell
function. Depending on the concentration, even routinely used probes can impact the system being measured (e.g. FITC-dextran is used to measure intralysosomal pH, but is also a weak acid that could potentially act as a buffer). Hence, the lower probe concentration required for a given application, the less likely the probe is to alter the system under observation, and extremely fluorescent probes suitable for use at low concentrations are very desirable. Data presented here clearly indicates that the extreme brightness of the CPNs allows use of very low labeling concentrations inside the cell. When combined with their low cytotoxicity, these evaluations clearly demonstrate the high utility of conjugated polymer nanoparticles as labels for both biological imaging and flow cytometry.

3.5 REFERENCES


   Doi:10.1371/journal.pone.0026626
4.1 INTRODUCTION

Conjugated polymer nanoparticles (CPNs) are formed by precipitation of highly fluorescent conjugated polymers [1,2]. CPNs largely retain the extremely bright fluorescence of their polymer component(s) found in other applications, such as thin films [3, 4], and as a consequence, have perhaps the brightest fluorescence per unit size of any characterized nanoparticle [5]. In addition, unlike many other nanoparticles that have detrimental effects on cellular function or morphology [6], CPNs have no observed cellular cytotoxicity [7, 9]. Their facile preparation, extreme brightness, easy uptake, and specific lysosomal delivery makes CPNs attractive fluid phase labels for range biological applications, including cellular imaging and flow cytometry experiments.

We have previously characterized cellular uptake of unmodified CPNs [7] and PEG-lipid functionalized CPNs [10] into macrophage cells, and carried out systematic comparisons of the intracellular brightness of CPNs and competing fluid-phase markers [10]. These studies demonstrate that CPNs are efficiently taken up into cells, and that their intracellular brightness is orders of magnitude brighter than competing fluid-phase markers, including quantum dots. Similar high relative intracellular brightness figures have been reported by other researchers [11]. Using cellular images and specific labeling
of lysosomal membranes, we have demonstrated that both bare and PEG-lipid coated CPNs are taken into macrophage cells via endocytosis, and are ultimately delivered to lysosomes. However, one aspect of our cellular uptake experiments was puzzling. Given the relative hydrophobicity of bare particles, we expected partitioning of bare nanoparticles into cellular membranes, or at the very least, substantial localization of CPNs on extracellular membranes that would be visualized as membrane localized fluorescence. However, cellular images showed no interaction of unmodified particles with the cell surface. Moreover, cellular inhibitor studies indicated that CPN uptake occurred via macropinocytosis, a mechanism that does not require binding at the cell surface [7]. In addition, while bare (unmodified) CPNs aggregate and precipitate under physiological salt conditions [12, 13], reflecting their high surface hydrophobicity, we observed no precipitation in our uptake experiments, which were carried out in serum solutions at physiological salt concentrations. The incongruity of these results with the expected hydrophobicity of unmodified CPNs led us to question whether the surface hydrophobicity of these CPNs was indeed as high as predicted in media or serum-containing solutions. We reasoned that CPNs in serum could be interacting with serum proteins. In this case, protein adsorption to otherwise unmodified CPNs would alter the CPN surface that was presented to the cells, and block interaction of a highly hydrophobic CPN surface with the hydrophobic cell membrane, as well as ameliorate aggregation. Hence, to understand CPN behavior under biological conditions, it is necessary to know whether unmodified CPNs in serum or media acquire a protein coat.
Interactions of proteins with the nanoparticle surface are well established for a variety of nanoparticle types [14, 15]. A recent review lists at least twenty nanoparticle types with demonstrated protein binding in solution [16]. Protein association includes a transient external layer (soft corona) and a long-lived inner layer (hard corona) that remains associated with nanoparticles after removal from protein solution(s) [17]. A series of researchers have isolated and identified the hard corona proteins interacting with individual nanoparticle types in serum or other biological fluids (recently reviewed in [15]). These investigations indicate that, for the nanoparticles studied, protein corona composition changes as a function of the nanoparticle type and size, reflecting changes in nanoparticle curvature, charge, or functional group orientation [18-20]. Corona composition is also somewhat sensitive to protein solution concentration and composition [21-24].

Notably, the presence or absence of the protein corona can affect nanoparticle biological activity. For a series of nanoparticle types, including polystyrene, iron oxide, and silica nanoparticles, cellular uptake and cytotoxicity is reduced by a protein coating [14, 25, 26]. Other nanoparticles can show increased uptake in the presence of a protein corona [27]. These data indicate that the recognition surface presented to cells in protein solutions reflects the protein coat, rather than the nanoparticle core characteristics [28]; the protein coat is the likely determinant of cellular uptake and/or efficiency, as well as of intracellular targeting. However, despite the importance of a potential protein corona on for biological applications of CPNs, no investigation of protein interaction with CPNs has been reported.
To test the interaction of CPNs with proteins in solution, we undertook a systematic study of the stability of unmodified PFBT CPNs under biological conditions in the presence and absence of added protein(s), including those found in serum. We demonstrate that under the conditions of high ionic strength used in physiologically relevant buffers and media, PFBT CPNs rapidly aggregate. However, when protein is included in the solution, bare particles acquire a stable protein coat. The resulting protein-coated particles have dramatically improved characteristics under biological conditions. Protein-coated CPNs do not aggregate, are stable over a wide pH range, and are taken into cells with greatly increased efficiency compared to either uncoated or PEGylated CPNs. These data demonstrate that protein adsorption is a facile and rapid method to modify otherwise hydrophobic CPNs surfaces, and will occur spontaneously in protein containing solutions, including serum. In addition, we demonstrate that adsorbed protein is available for molecular recognition. Protein adsorption thus both improves colloidal stability and offers a simple method to modify CPN surfaces with molecular recognition or targeting moieties.
4.2 MATERIALS AND METHODS

All chemicals and reagents used were commercially available. Polyfluorene conjugated polymer PFBT (poly[9,9-dioctylfluorenyl-2,7-diyl]- co-(1,4-benzo-{2,1’,3’}-thiadiazole)], (M<sub>w</sub> 48,000, polydispersity 2.7) (American Dye Source), HPLC grade Tetrahydrofuran, THF (Fischer chemicals), Bovine albumin serum (BSA) fraction V,(Omnipur), lysozyme (EMD), FBS (fetal bovine serum), Sulfo-NHS-biotin (Thermo Scientific) and streptavidin- magnetic beads (New England BioLabs) and FITC (Acros chemicals) were used without further purification. All water used was Barnstead Nanopure deionized water (18.2 MΩ) that had been further filtered through 20 nm PVDF filter (Whatman).

4.2.1 NANOPARTICLE PREPARATION AND PROTEIN ADSORPTION

Protein-modified PFBT CPNs were prepared by two different methods. In the first method, unmodified (bare) PFBT nanoparticles were prepared by reprecipitation, and then the resulting nanoparticles were incubated with protein in a separate step to allow protein adsorption to the CPN surface. Briefly, 1000 ppm PFBT polymer stock solution was diluted in tetrahydrofuran (THF) to make a 250 ppm solution that was rapidly diluted 1:10 in water (10-20 mL total volume) under continuous sonication with a micro-tip equipped sonicator (Branson; 45% power, corresponding to 67.5 W). Sonication was continued after dilution, to make a total of 2 minutes sonication. Following CPN formation, THF was removed by vacuum evaporation, and volume lost after evaporation
was replaced by addition of water. The nanoparticle suspension was finally filtered through a 220 nm PVDF membrane syringe filter. The resulting 25 ppm nanoparticle suspension was used for subsequent protein adsorption experiments. The nanoparticle molar concentration of 25 ppm PFBT was calculated to be 3±1 nM based on the mass of the conjugated polymer diluted in water during reprecipitation and the size of the CPN core as measured by TEM (Fig. S1). To allow interaction of protein with the bare PFBT CPN surface, bare nanoparticles were incubated with protein solution(s). Briefly, BSA was added directly to nanoparticles in water at different protein: CPN molar ratios; ranging from 33 to 250. Other proteins including biotinylated BSA (BSA-biotin), or lysozyme were added directly to nanoparticles in water at protein: CPN molar ratio of 83. Alternatively, to investigate interaction of total serum proteins with CPNs, 3±1 nM CPNs were incubated with 1% fetal bovine serum (FBS). After 1 hour of incubation with proteins, 2.5 ml of CPN-protein was mixed with 2.5 ml of 2x PBS (PBS:140mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4 at pH 7.2).

In the second (alternative) method, PFBT polymer in THF was diluted directly into water containing BSA, to create protein-modified PFBT CPNs in a single step. Briefly, BSA was mixed in 9 ml water, to make solutions with BSA, concentrations ranging from 100 nM to 750 nM when total volume is 10 ml. 250 ppm PFBT in 1 mL THF was diluted directly into each solution under continuous sonication, as described above, to create protein modified PFBT CPNs in a single step. THF was then removed in vacuum evaporation and lost solution mass was compensated by water addition. Similarly, 1 ml of 250 ppm PFBT in THF was dispersed directly in 9 ml of 1x PBS.
containing BSA in the concentration range from 100 nM to 750 nM. THF from the CPN suspension was removed by vacuum evaporation; volume lost was compensated with PBS, and finally filtered through 220 nm PVDF syringe filter.

PEG-Lipid incorporated PFBT CPNs were prepared by dispersing 1 ml of PFBT in THF into 9 ml of water containing 40 ppm PEG-Lipid methoxy 2000, as previously described [9]. During experiments to test for possible protein association with PEG-Lipid CPNs, 5.5 nM nanoparticles were incubated with 250 nM (final concentration) BSA in PBS for at least two hours prior to DLS measurement.

4.2.2 NANOPARTICLE SIZE AND ZETA POTENTIAL MEASUREMENT

The hydrodynamic size of the CPNs were measured and monitored by Photon Correlation Spectroscopy (PCS), also called Dynamic Light Scattering (DLS), using a Malvern Zetasizer (ZS90). DLS experiments were performed in the backscattering mode (scattering angle of 173°) at 25°C using distilled deionized water (refractive index 1.33) or PBS as dispersant wherever relevant. Refractive index of PFBT in THF at 25 ppm was 1.40 as measured using an Abbe refractometer. Prior to each measurement, CPN suspension were sonicated for 20 seconds in a bath sonicator to remove bubbles and minimize possible transitory/unstable aggregates. The Z-average mean hydrodynamic size and polydispersity index were determined and analyzed in terms of intensity weighted distributions using Cumulant analysis. Measurements were performed on each of three independent preparations; the mean and standard deviation were calculated from
measurement of three measurements each in duplicate. Zeta potential of CPNs were measured by Malvern Zetasizer(ZS90) using 10 mM TRIS buffer at pH 8.0 as dispersant.

4.2.3 NANOPARTICLE STABILITY STUDIES

To test the stability of bare PFBT CPNs in phosphate buffered saline (PBS), 1 ml of 25 ppm PFBT CPN in water was mixed 1 ml of 2X PBS. A similar experiment was performed in which equivalent amounts of CPN were added to 1 mL of 2X PBS containing 500 nM BSA, to make a 250 nM BSA solution in PBS. To test the pH-dependence of CPN stability, CPNs were diluted into pH calibration buffer (130 mM KCl, 15 mM HEPES and 15mM MES) at different pH (4 to 8). Each test solution was photographed under UV illumination by 365 nm handheld UV illuminator and CPN size was monitored by DLS (Malvern Zetasizer nano ZS) and fluorescence intensity measured by using steady state spectrfluorometer (Photon Technology International, PTI Quantmaster). The Emission spectra was collected using 460 nm excitation (2 nm band pass) and emission was scanned from (480-650) nm (1 nm step; 2 nm band pass).

The long term solution stability of the protein-modified particles stored in PBS at 4°C was monitored by DLS size measurement over a period of two weeks. CPN solutions were warmed to room temperature (25°C) prior to measurement, and DLS measurements were performed as described above.
4.2.4 OPTICAL PROPERTIES MEASUREMENT

Fluorescence spectra were acquired using steady state spectrofluorometer (Photon Technology International, PTI Quantmaster). The Emission spectra of CPNs were collected using 460 nm excitation (2 nm band pass) and emission were scanned from (480-650) nm (1 nm step; 2 nm band pass). Absorption spectra were measured using high resolution UV-vis spectrophotometer (UV-2501PC, Shimadzu). Quantum yield of CPNs was measured against fluorescein in 0.1M NaOH as standard using standard methods.

4.2.5 BIOTINYLATION OF BSA

Bovine albumin serum protein was biotinylated using Sulfo-NHS biotin following the manufacturer recommended protocol. Briefly, 60 µL of 10 mM biotin reagent solution was added in 1 ml of 2mg/ml BSA protein solution in PBS. The incubation reaction was carried out at room temperature for 30 minutes and excess biotin removed by size exclusion chromatography (Sephadex G50).

4.2.6 SIZE EXCLUSION CHROMATOGRAPHY

Excess protein in the solution was removed by size exclusion used to separate chromatography using a Sephacryl S-200 resin filled column with either water or PBS as eluent at flow rate of 1ml/min. Resulting CPNs in water or buffer were concentrated by centrifugal concentrator (Fisher Scientific AccuSpin1R).
4.2.7 MAGNETIC BEAD PULLDOWN

BSA-biotin coated PFBT nanoparticles (50 µL of 0.12 nM (1ppm) suspended in PBS buffer was added to streptavidin magnetic beads that had been extensively washed with Ringer’s Buffer (RB: 155mm NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 2 mM NaH₂PO₄, 10 mM glucose, 10 mM HEPES, pH 7.2-7.4). A mixture of magnetic beads and BSA-biotin coated CPNs were incubated together for 30 minutes at room temperature, before separation of magnetic beads under magnetic field. Separated beads were washed 5 x with RB to remove (any) unbound CPNs, then resuspended in RB to make a total of 150µL. 100 µL of this solution was diluted to 2500 µL in buffer and placed in a cuvette; the fluorescence emission spectra of PFBT CPNs captured by the magnetic beads were acquired using a photon counting spectrofluorometer. The fluorescence emission were collected from (480-650) nm using 460 nm excitation. Both excitation and emission monochromatic slits were set to achieve a 4 nm band pass. BSA-coated CPNs and PEG-Lipid methoxy 2000 incorporated CPNs were used as a negative control in this experiment.

4.2.8 CELL CULTURE

The J774 A.1 mouse macrophage like cells were grown in tissue culture flask using Dulbecco’s Modified Eagle Medium (DMEM; Mediatech) supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone), penicillin (100 units/mL), streptomycin (100µg/mL), and L-glutamine (2 mM) in a humidified incubator with 5%
CO2 at 37ºC until reaching to 70-80% confluence, then plated on glass bottom Petri dishes.

4.2.9 NANOPARTICLE UPTAKE

Cells were grown onto 35 mm tissue culture dishes. The cell culture medium was then replaced with serum free DMEM to remove serum proteins. BSA coated CPNs, lysozyme coated CPNs, bare CPNs, or PEG-lipid coated CPNs were added to dishes to a final concentration of 2 ppm/ ca. 0.24 nM and incubated for two hours to allow cell uptake (pulse), before DMEM in the extracellular solution was replaced with warm (37ºC) RB (without CPNs) and incubated for a further two hours (chase) before analysis of uptake. As a control, a similar experiment was performed on ice. In this case, BSA modified CPNs were incubated with J774 A.1 cells on ice for two hours (pulse), after which the DMEM was replaced with ice cold Ringer’s buffer and incubated for another two hours (chase). Following the 2 h chase, J774 A.1 cells were rinsed 3X with warm (37º C) RB, then suspended in ice cold RB by gentle pipetting. Cell-associated fluorescence was analyzed using a BD Accuri C6 flowcytometer, using 488 nm laser excitation and green fluorescence channel (FL1) for signal collection. 10,000 cells were analyzed for each measurement, and each sample was measured in triplicate. Data were analyzed using FlowJo software (Treestar), and uncertainty determined from the mean fluorescence of triplicate measurement for each sample.
4.3 RESULTS AND DISCUSSION

Protein adsorption prevents aggregation of otherwise unmodified PFBT nanoparticles at physiological salt concentration. We have prepared PFBT nanoparticles using the reprecipitation method [29], in which conjugated polymer in a small volume of THF are rapidly diluted into water under sonication. Under these conditions, the PFBT polymer chains rapidly fold to minimize hydrophobic surface area in contact with water, creating nanoparticles. After preparation and evaporative removal of THF, the resulting “bare” (unmodified) CPNs had a hydrodynamic diameter of 55±2 nm, as measured by intensity averaged cumulant analysis.

We believe that conversion of DLS intensities into nanoparticle hydrodynamic diameter using cumulant averaging (Z-averaging), as reported here, is the appropriate method for measurement hydrodynamic diameter, since it is rigorously supported by light scattering theoretical models. DLS diameters for nanoparticles are sometimes calculated from number averaged, rather than intensity averaged, scattering data. Unfortunately, transformation of light scattering into hydrodynamic diameter using number averaging is not supported by theoretical mathematical models. In addition, number averaging requires assumptions about refractive index that are rarely reported and if reported, are difficult to validate. For size comparison with reports of other CPNs measured via DLS number averaging, we have included hydrodynamic radii obtained from both cumulant intensity Z-averaging and number averaging (assumed polymer refractive index of 1.40, measured for soluble PFBT in THF) for these PFBT CPNs. As shown in Table 1, the number averaged hydrodynamic diameter is more than 20 nm smaller than that obtained
by cumulant intensity averaging, a result suggesting that use of number averaged DLS hydrodynamic diameter can lead to systematic underestimates of true hydrodynamic size. For example, we have observed that number averaged analysis lead to estimates of nanoparticle size that are 20-30 nm smaller than obtained by cumulant averaging (Table S1). Other size determination methods can also underestimate nanoparticle size. For example, we have previously reported that the PEG-Lipid PFBT CPN diameter is $24 \pm 5$ nm when dried and measured by TEM, while the same nanoparticles in suspension had a measured hydrodynamic size of $52 \pm 1$ nm when measured by intensity averaged scattering data [7] and $31$ nm diameter when analyzed via number averaged light scattering. We assume that smaller sizes reported by TEM relative to DLS reflect CPN shrinkage that occurs upon drying. AFM measurements also yield small apparent CPN sizes; PFBT CPNs prepared under conditions identical to those used here have reported AFM particle heights that vary from $14$ nm [30]or $18$ nm [31] to $25$ nm [32], all significantly smaller than measured by DLS intensity averaging. Very small particle heights obtained by AFM presumably reflect shrinkage during CPN drying, similar to that occurring during TEM, as well as compression of relatively soft CPNs by the microscopy tip during measurement. Based on a comparison of light scattering data for these and previously synthesized reprecipitated PFBT CPNs [7], we believe the bare PFBT CPNs described here to be sized consistently with the actual size of previously reported unmodified PFBT CPNs, despite apparent disparities between reported diameters.
We observed the DLS size of these bare PFBT particles in water over a period of 2 weeks, at a concentration of 10 ppm/1.2 nM. We observed no apparent change in hydrodynamic size (Figure S4), indicating that progressive aggregation does not occur in water in the absence of physiological salt concentrations. Similar results have been observed for other CPNs [12]. Given the high hydrophobicity of bare CPNs, formation of a long-lived nanoparticle suspension in water as observed here is somewhat unexpected [1]. Since removal of hydrophobic surface area from contact with water is sufficient to drive CPN formation, removal of additional hydrophobic surface area via CPN-CPN interactions (i.e. progressive aggregation) could also be expected to occur. However, it has been speculated that oxidation defects in individual conjugated polymer molecule starting materials could provide enough polarity to promote CPN solubility [1]. In addition, a recent study has demonstrated that oxidation defects are introduced into CPNs during vacuum removal of THF after CPN formation by reprecipitation [33], which would provide additional surface polarity. Local charges induced by chemical defects may thus be sufficient to explain both stability in water and the negative zeta potentials typically reported for unmodified CPNs [34].
Table 1. Comparison of size of PFBT CPNs measured by DLS, TEM and AFM. DLS size were measured in water for unmodified PFBT CPNs and PEGlipid\textsubscript{methoxy2000}\textsuperscript{-}PFBT CPNs; TEM size and AFM data were obtained as described in relevant reference.

<table>
<thead>
<tr>
<th></th>
<th>Unmodified PFBT CPN</th>
<th>PEGlipid\textsubscript{methoxy2000}\textsuperscript{-}PFBT CPNs</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS Z-average (nm)</td>
<td>55±2</td>
<td>58±1</td>
</tr>
<tr>
<td>Polydispersity Index</td>
<td>0.16</td>
<td>0.14</td>
</tr>
<tr>
<td>DLS number average (nm)</td>
<td>32±1</td>
<td>33±1</td>
</tr>
<tr>
<td>TEM size (nm)</td>
<td>N/D</td>
<td>24±5\textsuperscript{b}</td>
</tr>
<tr>
<td>AFM size (nm)</td>
<td>18±5\textsuperscript{a}</td>
<td>N/D</td>
</tr>
</tbody>
</table>


(b) Kandel et al Nanoscale, 2011, 3, 1037-1045.

To compare stability of the PFBT CPNs in water to stability under physiological conditions, we exchanged the CPNs into PBS, a buffer solution commonly used to approximate physiological conditions, and monitored CPN stability and aggregation over time. In this case, aggregation of CPN solutions can be monitored by tracking fluorescence intensity over time, since aggregation-induced precipitation and subsequent sedimentation reduces the concentration of fluorescent materials in the detection window of a cuvette. As shown in Figure 1A, the fluorescence associated with bare PFBT CPNs falls dramatically in PBS over a period of hours; loss of fluorescence is essentially complete after 12 hours. These results demonstrate that unmodified PFBT nanoparticles are not stable under conditions of physiological ionic strength, as has been observed
previously [12]. Unlike unmodified nanoparticles in water, unmodified CPNs in PBS aggregate and then precipitate. Hydrophobic aggregation of CPNs in solution mirrors commonly observed decreases in the solubility of hydrophobic small molecules and hydrophobic particles with increasing salt concentration [35]. Presumably, as the polarity of the solvent increases in the presence of higher salt concentrations, the hydrophobic driving force for aggregation also increases. At physiological salt, any local CPN surface polarity caused by chemical defects may be insufficient to counteract the increased hydrophobic driving force, resulting in enclosure of CPN surface area in aggregates.

![Fig.1. CPN fluorescence in PBS.](image)

**Fig.1. CPN fluorescence in PBS.** (A) Unmodified CPNs incubated in PBS; (B) Unmodified CPNs incubated in PBS containing 250 nM BSA. Reductions in fluorescence intensity reflect aggregation and precipitation out of the detection window. Inset shows the image of CPNs directly after dilution into PBS and after 12 hours, confirming that loss of fluorescence reflects CPN precipitation. Error bars represent the standard deviation of mean fluorescence.

Protein adsorption forms a hard corona that prevents CPN aggregation. We have previously prepared PFBT CPNs with a surface coating of PEG by inclusion of PEG
Lipid into the nanoparticle during conjugated polymer preparation. This methodology results in a high colloidal stability under physiological conditions [8]. Presumably, the resistance to hydrophobic aggregation we observed for PEGylated particles reflects shielding of the hydrophobic PFBT surface from the aqueous phase by a layer of PEG. In principle, any amphipathic polymer could perform a similar function. We asked whether proteins, which contain a mixture of polar and nonpolar regions, could also be used as a stable surface coating for CPNs. To test whether protein adsorption to CPNs improves colloidal stability under physiological conditions, we incubated prepared bare PFBT nanoparticles in solutions of bovine serum albumin (BSA) in PBS. BSA is the most abundant blood plasma proteins in mammals and is used extensively to reduce nonspecific binding via adsorption to hydrophobic surfaces in biological applications such as ELISA [36]. Like other proteins, BSA is a zwitterinoic polymer, whose hydrophobic amino acid side chains could be expected to interact with hydrophobic CPNs while its hydrophilic amino acid and carboxyl groups could interact with water and aqueous solution components. We added both BSA and physiological salt to suspensions of unmodified CPNs, and tracked the stability of CPN fluorescence over time. As shown in Figure 1B, the fluorescence intensity of CPNs in BSA-containing PBS is stable over at least 12 hours, indicating that under these conditions, the CPNs do not precipitate. This behavior is in sharp contrast to identical nanoparticles in PBS in the absence of BSA, which show profoundly decreased fluorescence at 12 hours as a result of aggregation and precipitation over the same time period.
We hypothesized that stability improvements in the presence of BSA reflected coating of hydrophobic nanoparticle surfaces via adsorption of hydrophobic regions on the protein. The resulting protein corona would presumably both stabilize the hydrophobic surface and acts as a barrier that occludes the hydrophobic CPN surface, with corresponding minimization of CPN-CPN interaction and resulting aggregation. To confirm our hypothesis of protein adsorption to the CPN surface, we compared the DLS size of CPNs before and after incubation with BSA. While bare PFBT nanoparticles have a DLS size of $55 \pm 2$ nm, CPNs incubated with BSA have a DLS size of $76 \pm 2$ nm, an increase of ca. 40%. Hydrodynamic diameter obtained via DLS is the average diameter of all dispersed objects, reflecting the average of the observed scattered light signal intensities. As a result, the calculated cumulant Z-averaged hydrodynamic size could be skewed by a bimodal (or multimodal) distribution of scattering that reflects contributions from small amounts of large particulates such as protein or CPN aggregates. Since larger particles (such as aggregates) have larger signal intensities, the presence of even small amounts of aggregate can result in substantially increased average nanoparticle size; it is correspondingly possible that the observed larger hydrodynamic diameter observed in BSA solutions could reflect the presence aggregates rather than size increases caused by a shell of adsorbed protein. However, this potential systematic error would be evidenced by a bimodal DLS trace; we observed a mono-modal light scattering distribution under the conditions of these experiments, indicating the absence of high MW aggregates (Figure S5A). In addition, DLS measurements of BSA solutions in the absence of CPN could detect no significant light scattering contribution from BSA at concentrations used
in the CPN incubations (data not shown). Hence, the ca. 20 nm observed increase in CPN hydrodynamic size following incubation of unmodified PFBT CPNs in BSA reflects increases in the size of individual CPNs caused by protein adsorption on the CPN surface. We note that the dimensions of fully folded BSA has been estimated at ca. 7 nm diameter [37], while fully or partially unfolded BSA would be somewhat larger. Assuming a BSA monolayer on the CPN surface, the 20 nm size increase observed for BSA-adsorbed CPNs thus implies that some BSA unfolding could occur to promote interaction of BSA hydrophobic regions with the CPN surface, consistent with previous reports of conformational changes in proteins adsorbed to other nanoparticle types [26, 38-40]. However, modeling of native BSA gives dimensions of 4 by 4 by 9 nm [41]; BSA interactions with CPNs exclusively along its short (4 nm) axis would give a BSA thickness virtually indistinguishable from the observed 20 nM size increase.

To compare the protein adsorption between bare and PEG-Lipid coated CPNs, we also incubated PEG Lipid-coated PFBT CPNs with BSA solutions. Since PEG is routinely used as a surface coating to ameliorate protein adsorption, we expected no significant interaction of PEG Lipid-coated CPNs with BSA. Indeed, the PEG Lipid-coated CPNs demonstrated no appreciable interaction with protein; PEG-Lipid-CPNs did not increase in size when incubated with BSA. PEG-Lipid coated PFBT CPNs had a DLS size of 58 nm in both PBS and BSA solutions, and was unchanged when monitored over 7 days (Figure S6).

To confirm that BSA adsorption to bare PFBT nanoparticles is primarily promoted by hydrophobic, rather than electrostatic, interaction(s), we observed both the
size and colloidal stability of PFBT CPNs in BSA solutions over the physiological pH range (from pH 4-8). Since BSA has a pI of 4.7, this pH range samples both protonated and unprotonated forms of the protein, with corresponding changes in net charge. If electrostatic interaction had the largest driving role in protein adsorption to the CPN surface, then the change in BSA charge with pH would significantly impact BSA interaction with the PFBT CPNs, and a change in the DLS size and/or colloidal stability between pH 4 and 6 would be observed. However, the size of BSA-adsorbed PFBT CPNs is largely independent of solution pH (Figure 2A), and no PFBT precipitation is observed in BSA-containing solutions at each of pH 4-8 (Figure 2B).
Fig. 2. BSA-CPN stability in high ionic strength buffer over the physiological pH range. (A) DLS hydrodynamic size in pH calibration buffers from 4-8; error bars represent standard deviation. (B) Images of CPN solutions at different pH values show no precipitation. BSA-CPNs were prepared by overnight incubation of unmodified PFBT CPNs in pH calibration buffer containing 250 nM BSA and measured without removal of excess BSA. Slightly larger hydrodynamic diameters observed here relative to particles in PBS or water may reflect the higher total ionic strength of pH calibration buffers relative to PBS, since protein conformation and hydrodynamic diameter are sensitive to ionic strength.

Together, these data indicate that adsorption of protein to CPN surfaces likely does not have a large electrostatic component, consistent with interactions driven primarily by the hydrophobic effect. Similar insensitivity to charge has been observed for BSA interaction with polymers on the surface of Au nanoparticles [42]. However, we cannot rule out some degree of electrostatic interaction with BSA; negative charges on the CPN surface caused by local chemical defects [33] could contribute to electrostatic
interaction with BSA, similar to what has been observed for electrostatic interaction of CPNs with positively charged polyelectrolyte copolymers in solution [43].

PFBT nanoparticles can be modified with a range of proteins. To test whether adsorption to the CPN surface reflects a specific interaction of BSA with PFBT, rather than a general hydrophobic adsorption phenomenon, we examined adsorption of additional proteins to bare PFBT particles. As part of these experiments, aliquots of unmodified PFBT nanoparticles were incubated in 250 nM solutions of BSA or lysozyme, or in 1% fetal bovine serum (which contains a mixture of serum proteins). The relative hydrodynamic sizes were measured to detect protein adsorption. These data show that adsorption to hydrophobic CPNs is not specific to BSA. As shown in Figure 3A, the hydrodynamic size of PFBT CPNs increased substantially following incubation in each protein solution. Further, the zeta potential of protein-coated particles is significantly less negative than bare particles (Figure 3B), presumably reflecting occlusion of surface negative charges from oxidative defects [33]. CPN zeta potential is less negative for lysozyme-coated particles than for those coated with BSA, consistent with the calculated charges of the respective proteins (lysozyme pI = 11.35; BSA pI = 4.7). Together, these alterations in CPN size and zeta potential of after incubation in proteins solutions indicate that a variety of proteins adsorb to the hydrophobic surface of CPNs, with resulting alteration of CPN properties.
Fig.3. Stable protein-coated CPNs can be prepared using different proteins. (A) hydrodynamic size of 3 nM CPNs incubated with 250 nM BSA, 1% Fetal bovine serum (FBS) or 250 nM lysozyme in PBS for 1 day (black bar), 3 days (light gray bar), or 7 days (dark gray bar). Error bars represent standard deviation. Prior to addition of proteins, bare CPNs had a DLS size of 55 nm. One way ANOVA analysis indicates that there is no significant difference in size in BSA (P= 0.412), FBS (P= 0.269), or lysozyme nanoparticle size over (P= 0.427) within group at various days, but statistically significant difference among treatment groups (P<0.001). (B) Zeta potential measurement of protein coated and bare CPNs in Tris buffer at pH 8.0. Error bars represents standard deviation of three independent measurements.

Notably, as shown in Figure 3A, the size of the CPNs incubated in different proteins/protein mixtures was not identical. The size difference between lysozyme-coated and BSA-coated CPNs could reflect differences in the efficiency of the two proteins’ interaction with the nanoparticle surface, as the accessibility and size of hydrophobic regions can vary between proteins. However, the size difference between lysozyme and BSA-coated CPNs likely also contributes to differences in CPN hydrodynamic diameter.
BSA is somewhat larger than lysosome (BSA MW 66,500 kDa and modeled native dimensions of 4 by 4 by 9 nm vs. lysozyme MW 14,300 and modeled native dimensions of 3 x 3 x 4.5 nm [41]), and BSA-coated CPNs may therefore be expected to have a somewhat thicker protein coating than lysozyme-coated particles, consistent with our observation. In contrast, CPNs incubated in FBS have size closer to BSA-coated CPNs. FBS contains a mixture of proteins, including a high concentration of BSA, and studies of the hard corona composition of a variety of nanoparticle types in serum show that BSA consistently binds nanoparticle surfaces. As a result, BSA could be a primary contributor to the protein corona in FBS solution(s). However, protein corona(s) in complex protein in serum typically contain a large number of different adsorbed proteins [15]), and it is likely that BSA is not the only component of the CPN corona in FBS solutions.

We note that CPN size increases are on the large end of what would be expected from a monolayer of native protein. However, interaction with nanoparticles often induces some degree of protein unfolding [40], which could allow extended protein conformations. Indeed, since hydrophobic regions in folded proteins are disproportionally located in the protein interior, it is plausible to assume that hydrophobic adsorption to CPN surfaces would be accompanied by local unfolding of protein structure to allow interaction of interior hydrophobic regions with CPNs.
Table 2. Hydrodynamic size of PFBT-BSA CPNs prepared by incubating BSA with CPNs after CPN formation. BSA was added in the mentioned ratio of BSA to CPN and incubated overnight before size of particles was measured by DLS.

<table>
<thead>
<tr>
<th>Molar concentrations BSA:CPN (nM)</th>
<th>Molar ratio BSA:CPN</th>
<th>Hydrodynamic diameter of PFBT CPNs in BSA/water (nm)</th>
<th>PDI</th>
<th>Hydrodynamic diameter of PFBT CPNs in BSA/PBS (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>750:3</td>
<td>250</td>
<td>(bimodal trace)</td>
<td></td>
<td>(bimodal trace)</td>
<td></td>
</tr>
<tr>
<td>500:3</td>
<td>166</td>
<td>(bimodal trace)</td>
<td></td>
<td>(bimodal trace)</td>
<td></td>
</tr>
<tr>
<td>375:3</td>
<td>125</td>
<td>77±2</td>
<td>0.19</td>
<td>78±2</td>
<td>0.18</td>
</tr>
<tr>
<td>250:3</td>
<td>83</td>
<td>76±2</td>
<td>0.17</td>
<td>76±1</td>
<td>0.16</td>
</tr>
<tr>
<td>200:3</td>
<td>66</td>
<td>68±3</td>
<td>0.15</td>
<td>78±1</td>
<td>0.18</td>
</tr>
<tr>
<td>150:3</td>
<td>50</td>
<td>65±2</td>
<td>0.16</td>
<td>(multimodal trace)</td>
<td></td>
</tr>
<tr>
<td>100:3</td>
<td>33</td>
<td>60±2</td>
<td>0.19</td>
<td>(multimodal trace)</td>
<td></td>
</tr>
</tbody>
</table>

BSA adsorption to CPN surfaces presumably reflects both the affinity of the protein for the CPN surface and the concentration of protein in solution. In the absence of sufficient protein in solution, the CPN surface could be inadequately covered, and CPN aggregation could still occur. To determine the amount of solution BSA required to completely coat CPN surface, various molar ratios of protein/CPN were tested. Molar ratios (protein/CPN) less than ca. 50 resulted in aggregation under physiological salt conditions.
conditions, as evidenced by increased DLS size and polydispersity induces in PBS. (Table 2). In this case, aggregation at low BSA concentrations presumably reflects an incomplete protein coating that is not sufficient to prevent all aggregation. We conclude that as long as the protein/CPN molar ratio in the incubating solution was above ca. 80, the PFBT CPNs were coated with a complete “corona” of BSA. For BSA solutions, molar ratios more than ca. 170 resulted in a small bimodal peak in DLS (Figure S3), which could reflect aggregation caused by BSA dimerization at high concentration.

**Fig.4 Protein coated CPNs retain their protein coat after removal of solution-phase proteins.** (A) DLS size of BSA-coated CPNs in the presence of solution phase BSA (left hand side; black bars) or after size exclusion chromatography for removal of solution phase BSA (right hand side; gray bars). (B) DLS size of lysozyme-coated CPNs in the presence of solution phase lysozyme solutions (left hand side; black bars) or after size exclusion chromatography to remove solution phase lysozyme (right hand side; gray bars). Prior to size exclusion, protein-coated NPs were prepared by incubation of 3 nM bare (55 nm) CPNs with 250 nM protein. All protein coated CPNs were suspended and stored in PBS. One way ANOVA analysis indicates that there is no significant difference in mean size before and after size exclusion for either BSA coated particles (P=0.064) or lysozyme coated particles (P=0.412).
Protein adsorption to CPN surfaces is not reversible under normal incubation conditions. So-called “hard corona” proteins remain associated with nanoparticles following removal from protein solutions, while weaker affinity “soft corona” proteins dissociate as a function of low protein concentration, and are not retained under conditions of dilution or washing [17]. To determine whether the adsorbed proteins coating PFBT CPNs reflects a long-lived coating surrounding the nanoparticle, similar to the “hard corona” characterized for other nanoparticle types, we used size exclusion chromatography to separate BSA-coated and lysozyme-coated CPNs from excess protein in solution. We did not separate FBS protein-coated CPNs from excess proteins in solution, as large variation in protein sizes in this complex solution made the separation impractical. A comparison of the DLS size of protein-coated CPNs before and after size exclusion into PBS (Figure 4) shows no change in hydrodynamic diameter after removal of solution-phase protein for both BSA-coated and lysozyme-coated CPNs. Moreover, the constant size and resistance to precipitation in PBS persisted over the period of observation (7 days). These data indicate that the protein-PFBT interaction does not dissociate even under conditions of prolonged incubation in the absence of excess protein. Both BSA and lysozyme association with PFBT CPNs is therefore sufficiently stable to be considered part of a hard corona, and to persist following removal to alternate solution conditions with dramatically lower protein concentration. In order for protein adsorption to be useful as a surface modification technique, the protein coating must be long-lived under physiological conditions, and not dissociate from the CPN. These data indicate that BSA and lysozyme adsorption to PFBT CPNs meets these conditions.
Table 3. Comparison of CPN size and spectral properties. Unless otherwise specified, all CPN protein coats were formed by incubation of unmodified CPNs in relevant protein solutions and unbound protein was removed by size exclusion chromatography; all spectral properties are measured in water.

<table>
<thead>
<tr>
<th></th>
<th>Z-average hydrodynamic diameter (nm)</th>
<th>Quantum Yield (%)</th>
<th>$\lambda_{\text{max}}$ absorption (nm)</th>
<th>$\lambda_{\text{max}}$ emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmodified PFBT CPN</td>
<td>55±2 (PDI 0.16)</td>
<td>13.6±0.8</td>
<td>462</td>
<td>537</td>
</tr>
<tr>
<td>BSA-CPNs (coat formed by incubation with BSA)</td>
<td>76±2 (PDI 0.16)</td>
<td>16.2±0.8</td>
<td>458</td>
<td>535</td>
</tr>
<tr>
<td>lysozyme-CPNs</td>
<td>71±1 (PDI 0.15)</td>
<td>14.2±0.6</td>
<td>460</td>
<td>536</td>
</tr>
<tr>
<td>FBS-CPNs</td>
<td>75±1 (PDI 0.18)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PEG Lipo$\text{ methoxy}_{2000}$-CPNs</td>
<td>58±1 (PDI 0.14)</td>
<td>21±1</td>
<td>459</td>
<td>535</td>
</tr>
<tr>
<td>BSA-CPNs (coat formed via reprecipitation into BSA)</td>
<td>78±2 (PDI 0.18)</td>
<td>15.5±0.8</td>
<td>458</td>
<td>535</td>
</tr>
</tbody>
</table>

CPN optical properties are not hampered by protein adsorption. To examine the effects of a protein corona on CPN optical characteristics, we compared the absorbance and emission maxima and quantum yields of bare and BSA-coated PFBT CPNs. Quantum yields for bare and protein-coated PFBT CPNs are shown in Table 3; data for PEG-Lipid modified PFBT particles are also included for reference. While bare PFBT
CPNs in water have a quantum yield of 13.6 ± 0.8 %, the same particles incubated in BSA have a slightly higher quantum yield (16.2 ± 0.8%). These data show that protein adsorption does not impede, and in fact can improve CPN brightness.

We have previously observed higher quantum yields for PEG Lipid-modified PFBT CPNs than for bare CPNs, and have attributed this increase in quantum yield to insertion of lipid between polymer chains in the CPN core, consistent with a small blue shift in absorbance maximum relative to unmodified particles [8]. In this case, insertion between polymer chains presumably disrupts the intrachain quenching observed for tightly collapsed conjugated polymer [44] and results in somewhat higher observed quantum yields. To determine whether insertion of hydrophobic moieties into the PFBT core could be responsible for the increased quantum yields for protein-coated CPNs, we compared the absorption spectra of bare and BSA-coated particles (Figure S7). A small blue shift of 4 nm is observed for BSA-coated PFBT CPNs compared to unmodified particles. Hence, the increased quantum yield may result from small disruptions in PFBT intra-chain interaction caused by insertion of hydrophobic protein groups (presumably hydrophobic side chains) between segments of PFBT chain(s) in the CPN core.

We note that we have previously reported a slightly higher quantum yield for bare PFBT CPNs (18 ± 1 % versus 13.6 ± 0.8 %). These previously described PFBT CPNs were synthesized from a different manufacturer’s lot of PFBT, and differences in optical properties presumably reflect differences in the PFBT starting material. Optical data reported here was the average of three consecutive independent syntheses of PFBT
CPNs, and show that for a given lot of PFBT, the reprecipitation method gives highly reproducible optical characteristics for each surface modification/coating.

Protein-modified CPNs can be prepared either by incubation in, or polymer dilution into protein solutions. Data shown above demonstrate that bare (unmodified) CPNs prepared by reprecipitation into water can be subsequently incubated in BSA solutions to produce BSA-modified CPNs. We have previously prepared CPNs with a PEG-lipid surface by reprecipitation in water containing PEG lipid. We asked whether BSA-modified CPNs could be prepared similarly prepared (i.e. by reprecipitation into BSA containing solutions). In this case, BSA would partition into the hydrophobic phase (i.e. the PFBT) during conjugated polymer precipitation. To test the efficacy of this alternate method of BSA-modified CPNs, we prepared PFBT nanoparticles via reprecipitation of PFBT into water containing BSA. Resulting nanoparticles had a DLS size that was indistinguishable from that obtained by incubation of unmodified particles in BSA solutions (78 ± 2nm versus 76 ± 2), and did not change when observed over 5 days. Fluorescence quantum yield was indistinguishable from that for BSA-coated CPNs obtained via incubation of bare CPNs in BSA (Table 2). The dependence of CPN hydrodynamic size on PFBT: BSA ratio was also indistinguishable from that obtained for incubation of bare CPNs in BSA (Table S2). These observations indicate that reprecipitation in the presence of BSA or incubation with BSA after reprecipitation into water is equally efficient methods for generation of BSA-coated PFBT CPNs. We note that dilution of conjugated polymer (in THF) into BSA solutions exposes the BSA to THF, which does not favor native protein structure. Incubation of bare particles with
protein solutions may therefore be the desired protein adsorption protocol if preservation of native protein folding is crucial. However, since studies of the conformation of proteins adsorbed on many different nanoparticles types demonstrate that protein-nanoparticle interactions can themselves induce local protein unfolding [26, 38, 39], any negative impact of possible THF-promoted protein unfolding is likely insignificant.

Protein adsorption can be used to modify CPNs for molecular recognition. To further establish that protein adsorption to CPNs results in a coating on the CPN surface, and to determine whether that protein coating could be used for bioconjugation, we performed pulldown experiments using biotin-BSA coated PFBT CPNs and streptavidin magnetic beads. Biotin is useful as a tether to attach additional recognition moieties via the high affinity biotin-streptavidin interaction. We prepared BSA-biotin coated PFBT CPNs by adsorption of bare CPNs with BSA-biotin, using protocols similar to those established for BSA adsorption. Excess BSA-biotin was removed from the CPN solution using size exclusion chromatography. Streptavidin magnetic beads were then incubated with a suspension of biotin-BSA CPNs to allow biotin-streptavidin binding. The magnetic beads were then removed from solution, washed, and resuspended in additional buffer. As shown in Figure 5, a fluorescence scan of the suspended magnetic beads shows substantial PFBT fluorescence, indicating capture of biotin-BSA by streptavidin. No PFBT fluorescence was observed when the same experiment was performed with either PEG Lipid-coated CPNs or BSA coated CPNs which lack the biotin recognition moiety. These controls indicate that CPN fluorescence observed for capture of biotin-BSA-modified PFBT CPNs reflects specific binding of biotin-BSA by the streptavidin beads.
Together, these data indicate that surface functionalization of CPNs by protein adsorption can be used for conjugation of relevant recognition moieties to the CPN surface; protein surface modification of CPNs not only provides solution stability but also a handle for bioconjugation.

**Fig. 5 Streptavidin-magnetic beads capture biotin-BSA modified CPNs.** Fluorescence emission reflects the presence of BSA-biotin coated CPNs in the solution. Streptavidin magnetic beads were exposed to a solution containing biotin-BSA-coated CPNs, followed by magnetic bead removal, washing, suspension of beads in fresh buffer, and scanning of fluorescence emission associated with magnetic beads. Significant CPN fluorescence is associated with beads incubated with biotin–BSA coated PFBT CPNs (solid line), while beads incubated with either PEG lipid-coated PFBT CPNs (dotted line) or BSA-coated CPNs (dashed line) show no detectable conjugated polymer fluorescence.

Protein adsorption substantially increases uptake efficiency. To determine the biological impact of a protein corona on CPN uptake in mammalian cells, we used flow cytometry to measure the uptake of PFBT CPNs with several different protein coronas
(BSA, lysozyme, and serum proteins). To compare these data with our previous uptake experiments, we also observed uptake of PFBT CPNs modified with PEG-Lipid. Since we have demonstrated that these PEGylated particles do not change size in the presence of BSA (Figure S4), PEG-Lipid PFBT CPN uptake represents uptake in the absence of a protein corona and serves as a negative control. Cells were incubated with the different nanoparticles in protein-free media to allow uptake, followed by washing of cells to remove any nanoparticles associated with cells, and the fluorescence associated with each cell was counted by flow cytometry. As shown in Figure 6, more than twelve-fold higher fluorescence was observed for cells treated with BSA-coated CPNs than cells treated with PEG-lipid modified CPNs. Increased cell-associated fluorescence was also observed for lysozyme- and serum coated CPNs relative to PEG Lipid particles. Since total fluorescence associated with flow cytometry measurements cannot distinguish between CPNs taken into cells and those simply localized at the cell surface, we also performed uptake experiments in which BSA-coated PFBT CPNs were incubated with cells on ice. At these low temperatures, energy dependent cellular uptake (i.e. endocytosis) does not occur, and any CPN fluorescence associated with the cell must reflect adsorption to the cell membrane, and/or (less likely) diffusion through cellular membranes. Hence, the difference between cell-associated fluorescence at physiological conditions and on ice reflects cellular uptake via endocytosis. As shown in Figure 6, little cell-associated fluorescence is observed for cells incubated with BSA-coated PFBT CPNs on ice, indicating that the majority of cell-associated fluorescence reflects energy-dependent uptake of the CPNs into the interior of the cell. Taken together, these data demonstrate
that the presence of a protein corona substantially increases cellular uptake. The efficiency of uptake is reflects both the presence and composition of the protein corona, since the uptake efficiency varies both between protein-coated and PEG Lipid-coated CPNs, and between CPNs coated with different proteins.

**Fig. 6 Cellular uptake.** Cells in serum free media were incubated with (left to right) unmodified PFBT CPNs; PEG-lipid PFBT CPNs, BSA-coated PFBT CPNs, lysozyme-coated PFBT CPNs at 37°C or with BSA-coated PFBT CPNs on ice (right hand bar). Following incubation, cells were exchanged into CPN-free Ringer’s buffer and allowed to incubate for an additional 2 h before total CPN fluorescence associated with the cells was determined by flow cytometry. Error bars represent the standard deviation of the mean fluorescence at 488 nm excitation. 10,000 cells were counted in each measurement.

We also examined the uptake of bare CPNs incubated in protein-free media. Interestingly, these unmodified particles also showed higher uptake than that observed for PEG-Lipid CPNs (Figure 6). In addition, although unmodified particles would be
expected to aggregate under the high ionic strength solution conditions found in media (similar to those observed in PBS), we observed no signs of CPN aggregation or precipitation in the solution conditions of the cellular uptake experiments. Indeed, while bare CPNs in PBS aggregate and precipitate in less than two hours, as observed by fluorescence intensity changes, bare CPNs in DMEM show no difference in fluorescence intensity for at least six hours, although precipitation was observed at a 24 h time point. These data suggest that in the absence of proteins, CPNs in media interact with DMEM components in ways that increase CPN stability to aggregation and improve cellular uptake, albeit with substantially less impact on colloidal stability than offered by whole protein adsorption. We examined the components of DMEM for the presence of hydrophobic or amphiphilic components that could be interacting with the bare CPNs under these conditions. DMEM contains a significant concentration of hydrophobic vitamins and amino acids, including those with hydrophobic side chains. We speculate that hydrophobic adsorption of these amphiphilic molecules to CPNs is responsible for the improved colloidal stability and higher uptake than would be otherwise expected.

4.3.1 DISCUSSION

Protein adsorption can be a surface modification strategy. Data included here demonstrates that while bare CPNs precipitate under physiological salt conditions, CPNs modified with a layer of adsorbed protein are stable for long periods. Stable protein-modified CPNs coated with a protein layer can be generated either by incubation of bare CPNs with protein solutions, or by reprecipitation of conjugated polymers into protein-
containing solutions; we demonstrated protein adsorption of CPNs with BSA, lysozyme, and serum proteins present in fetal bovine serum. Together, these data show that proteins form a stable corona at the CPN surface that persists even after long incubation in protein-free solutions. This result, although not previously documented for CPNs, is not unexpected, given the high hydrophobicity of CPNs and the well-documented interactions of proteins with other hydrophobic nanoparticle types [15]. Our data demonstrate that for CPNs, protein adsorption provides substantial long-term increases in colloidal stability. The observed increase in CPN stability with protein adsorption is different than the behavior of nanoparticles whose formation and stability results from electrostatic interactions, such as DNA lipoplexes, which can be destabilized in protein solutions [45].

Competing methods for protecting CPNs against aggregation under physiological conditions chiefly involve incorporation of PEG, PEG-like compounds, or other amphiphiles into the nanoparticle surface during CPN formation [11, 12, 46, 47]. For example, we have previously demonstrated that extremely stable PFBT CPNs coated with PEG can be synthesized by rapid dilution of conjugated polymers into PEG-lipid solutions. These PEG coated CPNs resist hydrophobic aggregation over long periods, even at high concentrations [8]. However, attempts to stabilize CPNs with a PEG coating are not universally successful; Mark Green’s laboratory uses the miniemulsion method to produce PEG coated MEH-PPV CPNs that still show signs of aggregation in PBS [13]. Similarly, Daniel Chiu’s laboratory has produced PEGylated CPNs, obtained via entrapment of polystyrene functionalized with PEG during CPN synthesis, are reported to
require an additional BSA passivation step to prevent protein adsorption [11], an observation that suggests that PEG moieties do not completely occlude hydrophobic surface area in these particles. Data shown here indicates that protein adsorption to CPNs can be used instead of PEGylation as a straightforward approach to promote long term colloidal stability.

Our data indicate that protein interaction with bare CPNs likely occurs via hydrophobic adsorption. Hydrophobic adsorption as a CPN surface modification method has been disparaged in a recent review [2], where the authors argue that surface coatings created by hydrophobic adsorption can dissociate under extended incubation and are therefore undesirable. This argument is somewhat surprising, given the accepted use of hydrophobic adsorption as a method for quantum dot surface modification [48] and the centrality of hydrophobic adsorption as the driving force for CPN formation. Data included here demonstrate that PFBT CPNs coated with protein via hydrophobic adsorption can be exchanged into protein-free solutions without dissociation of the protein coating, even after extended incubation. Hence, hydrophobic adsorption of proteins to CPNs produces highly stable surface modification that does not readily dissociate. This observation is similar to studies of protein encapsulated polystyrene nanoparticles, in which protein adsorption to the hydrophobic polystyrene surface has been described as “essentially irreversible” [17] and studies of interaction of conjugated polymer films with BSA which demonstrated that adsorbed protein was retained even in the presence of SDS [49]. We conclude that hydrophobic adsorption is highly suitable for CPN modification. Notably, since we have demonstrated that protein rapidly adsorbs to
CPN surfaces during incubation, we speculate that at least some of the previous reports of covalent protein attachment to CPNs may in fact reflect non-covalent interactions. Data shown here indicate that significant controls would be required to distinguish between covalent attachment and stable protein adsorption, particularly for conjugation protocols which incubate protein with CPNs prior to addition of conjugation reagents.

The Chiu group has incubated CPNs with the amphiphilic polymers poly (styrene sulfonate) and poly (sodium methacrylate) as a method to both increase colloidal stability of CPNs and allow bioconjugation [12]. After incubation, the CPNs were reported to be coated with a shallow (2 nm) layer of polymeric polyelectrolyte, presumably tethered to the PFBT surface primarily by hydrophobic adsorption. The resulting coated PFBT particles were highly stable in salt solutions, including PBS, a result which corroborates the viability of adsorption of polymeric amphiphiles (including proteins) as method for increasing CPN colloidal stability. The authors then used the adsorbed coating as the foundation for covalent conjugation to protein that was finally utilized, in an additional step, for molecular recognition. As an alternative, we have used BSA-biotin as a CPN coating material that both increases colloidal stability and can be used to tether streptavidin. Together with other results included in this paper, these data demonstrate that protein adsorbed on the CPN surface (1) can be substituted for the amphiphilic polymers used by Chiu to increase colloidal stability; (2) remains tethered to the CPN surface without requirement for covalent attachment; (3) is available for molecular recognition, and (4) can be included on the CPN surface during reprecipitation. Hence,
we are able to generate stable protein-coated CPNs suitable for molecular recognition processes, including bioconjugation, in a single step.

4.4.1 IMPLICATIONS OF PROTEIN CORONAS ON CPNs FOR BIOLOGICAL APPLICATIONS

Our data demonstrate that in biological protein-containing solutions such as serum or plasma, bare CPNs do not maintain their conjugated polymer surface, but are coated with a stable layer of protein. Hence, the CPN presented to the cell for uptake in biological media does not resemble a bare particle. First, the nanoparticle is substantially larger than sizes reported for bare particles; while bare PFBT CPNs have an AFM size of 18 nm and a DLS intensity averaged size of 55 nm [7] in water, they measure 75 nm in the fetal bovine serum solutions typically used for cellular uptake and imaging studies. Repeated claims have been made about the necessity of small particles for efficient uptake, with a focus on nanoparticles smaller than 30 nm [2]. Our data suggest that a protein coat is likely to add 18 to 25 nm to DLS intensity-averaged hydrodynamic size, and substantially more to sizes reported by TEM diameter or AFM particle height. Hence, few (if any) unmodified CPN particles prepared are likely to be smaller than 30 nm in physiological protein solutions such as serum or plasma. Larger sizes are not a barrier to cellular uptake via endocytic processes, as we have demonstrated, but are likely to prevent passive diffusion through (ca. 9 nm) [51] nuclear pores and the (ca. 4 nm) [52] tight epithelial junctions between cells.
Our data demonstrate that it is the adsorbed protein corona that determines cellular uptake, not the conjugated polymer core characteristics. PFBT CPNs coated with protein have more than an order of magnitude higher cellular uptake than observed for PFBT CPNs lacking a corona (i.e. PEG Lipid-modified PFBT CPNs). Hence, the protein coat presented to the cell determines uptake efficiency. The observed impact of a protein corona on CPN uptake has implications for the intracellular brightness of CPNs compared to competing labels. We have previously used direct comparisons of the intracellular brightness of PEG Lipid-modified PFBT CPNs, Q-dots, and dextran-linked fluorescent dyes to conclude that CPNs are 37 times brighter than Q dots and 600 times brighter than fluorescent dyes when used at equivalent concentrations as intracellular fluid phase labels. However, since intracellular brightness is partly a function of uptake efficiency, and protein-coated CPNs are taken into cells with at least an order magnitude higher efficiency than the PEG-Lipid PFBT CPNs, we can correct these brightness estimates to include the impact of increased uptake in the presence of a protein corona. Notably, these modified intracellular brightness estimates suggest that in the presence of a protein corona, the relative intracellular brightness of protein-coated CPNs is ca. 400 times higher than that of a representative quantum dot, and at least 6000 times higher than dextran-linked fluorescent dyes.

Formation of protein corona surrounding CPNs in biological solutions has additional implications for cellular targeting strategies. In particular, recognition moieties tethered to the CPN core can be occluded by formation of a protein coat in serum, as has been observed for transferrin-functionalized silica nanoparticles [53]. Hence, formation
of a possible protein corona must be considered when designing CPNs for biological applications, particularly for strategies that involve conjugation of small targeting groups, such as a peptides, or small tethering agents, like biotin. While our PEG Lipid modified PFBT CPNs [8] do not measurably adsorb protein(s) and would behave well under these circumstances, protein adsorption could be a potential complication for other CPNs, including those with a PEG-like surface. For example, an alternative PEG-Polystyrene comb-coated PFBT CPN was reported to bind “nonspecifically” to streptavidin (i.e. without the presence of biotin) unless incubated in BSA [11]; in other words, this nanoparticle type adsorbs protein(s) in solution that could occlude small targeting moieties such as peptides.

One way to bypass complications from protein interference with molecular recognition strategies is to use the protein corona itself as the foundation for bioconjugation. We have demonstrated that adsorption of biotinylated BSA to PFBT CPNs allows capture of the nanoparticles by streptavidin beads. Hence, moieties associated with adsorbed protein are accessible for molecular recognition. Protein adsorption thus creates an easy and convenient platform for CPN functionalization. For example, CPNs adsorbed with BSA-biotin could be linked to any avidin or streptavidin-linked surface. Similarly, adsorption of streptavidin could be used to functionalize with biotin-linked nucleic acid or peptide moieties, while IgG-adsorbed CPNs could be utilized for detection in immunoassays.
4.4 CONCLUSION

Taken together, data included here clearly demonstrates that the presence or absence of a protein corona surrounding CPNs in solution has important implications both for colloidal stability and for the behavior CPNs in biological applications, consistent with what has been reported for other nanoparticle types [15, 28]. In the absence of strategies to inhibit protein interaction with unmodified CPNs, the surface presented to cells is defined by the protein corona, with corresponding impact(s) on cellular uptake. Hence, no discussion of the biological applications of CPNs can be carried out independently of discussions of protein adsorption. However, if the protein coating is defined by deliberate formation of protein-modified CPNs, as demonstrated here, then a protein corona may be used as a flexible tool for bioconjugation, rather than an experimental complication.

4.5 REFERENCES


4.6 SUPPLEMENTAL FIGURES

**Fig. S3. Histogram of size distribution of measured PFBT-BSA CPNs diameter.** Image was analyzed using Image J software. Gaussian fit to histogram shows the average diameter to be $30 \pm 2$ nm (mean ±SD).

**Fig. S4. Hydrodynamic Diameter of unmodified PFBT particles in water over time.** Size was determined by Z-average cumulant analysis of DLS data. CPN concentration was ca. 3 nM. Error bars represent standard deviation of three independent measurements. One way ANOVA indicates that there is no statistically significant difference between measured sizes over time ($P = 0.236$).
Fig. S5 Monomodal and bimodal light scattering of suspensions of BSA-CPNs. (A) DLS trace at 3.0 nM CPN: 250 nM BSA in water. (B) DLS trace at 3.0 nM CPN: 500 nM BSA in water.

Fig. S6. PEG Lipid-PFBT CPNs in presence of BSA. Bar plot shows hydrodynamic size determined by DLS Z- average cumulant analysis over a period of one week. Black bars: 5 nM PEG lipid–PFBT CPNs incubated in PBS alone; Gray bars: 5 nM PEG Lipid-PFBT CPNs in PBS with addition of 250 nM BSA. One way ANOVA analysis indicates that there is no statistically difference in size for PEG Lipid-PFBT CPNs in PBS and in PBS with BSA (P=0.262)
Fig. S7. Coating PFBT with BSA results in blue shift. Absorbance spectra of bare PFBT CPNs (dotted line) with absorbance maximum at 462 nm and BSA-coated PFBT CPNs (solid line) with absorbance maximum at 458 nm in water. BSA-coated CPNs were formed by incubation of 3 nM CPNs with 250 nM BSA, followed by size exclusion into water to remove non-bound BSA.

Fig. S8. Size Exclusion separation of BSA-CPNs from excess BSA. Solid line shows PFBT-BSA normalized absorbance at 460 nm; dotted line shows normalized BSA absorbance at 280 nm. Retention time for BSA-coated PFBT CPNs is 15 ± 3 minutes (FWHM), and for BSA alone is 21±2 minutes (FWHM).
CHAPTER FIVE

FUNCTIONALIZED CONJUGATED POLYMER NANOPARTICLES BY PROTEIN ENCAPSULATION AS DETECTION REAGENTS IN SOLID PHASE IMMUNOASSAY

5.1 INTRODUCTION

Conjugated polymer nanoparticles (CPNs) are formed by condensation of hydrophobic conjugated polymers with ultra-bright fluorescence. The resulting nanoparticles have among the brightest fluorescence per unit size of all characterized nanoparticles [1], with no observable cytotoxicity [2-4]. Comparisons of the intracellular brightness of CPNs with quantum dots and small molecule fluorescent dyes estimate that these nanoparticles are (30-40)-fold and 600–fold brighter, respectively, than equimolar amounts of either competing label when taken into cells, depending on the individual CPN and cell type [5,6]. As a result, these nanoparticles have excited much interest as fluorescent labels for cellular imaging, flow cytometry [7], and highly sensitive diagnostics. We have demonstrated that both bare and PEG Lipid-coated CPNs are efficiently taken into macrophage cells by macropinocytosis and trafficked to the lysosome [2,3]. Importantly, lysosomal trafficking enables use of CPNs as ultra-bright fluid-phase labels [5].

If CPNs are to be used beyond fluid phase labeling, however, the CPN surface must be modified with relevant biorecognition moieties. For example, CPNs used as labels for nucleic acid hybridization assays must be linked to a specific nucleic acid
oligomer; CPNs used for highly sensitive immunodiagnostic detection must be linked to specific antibodies. In addition, CPNs could also be applied as bright labels for individual cellular locations in live cell imaging if they were bioconjugated to targeting moieties, including peptide signal sequences that promote trafficking to specific intracellular organelle types, antibodies or proteins that interact with specific cell-surface receptors to promote trafficking via known endocytic mechanisms [8-10], or known cell penetrating peptides[11-13]. Hence, surface modification of CPNs is a prerequisite for effective and widespread bioanalytical application. Unfortunately, unmodified CPNs that contain only conjugated polymer are not readily amenable to chemical modification, which makes direct attachment of targeting moieties problematic. In addition, the highly hydrophobic surface of bare CPNs leads to aggregation under physiological salt conditions [14-15], which impedes their application in diagnostics that require physiological buffers. Hence, much effort has been expended to generate methods for CPN surface modification as a path to both bioconjugation and improved colloidal stability [3, 6, 7, 15-23].

Initial surface modifications strategies focused on inclusion of PEG on the CPN surface to promote colloidal stability and allow covalent attachment of additional functional groups via the reactive endgroup. For example, we have demonstrated that the biotin endgroup of PEG on the surface of reprecipitated PEG Lipid-CPNs can be used to for tethering of streptavidin–linked antibodies, and have used that interaction for specific labeling of cell-surface receptors [3]. Similarly, Howes et al and others have reported covalent linkage of a protein to carboxy endgroups present on the PEG Lipid functional group of CPNs synthesized by miniemulsion [17, 22]. Instead of using PEG-Lipid, Koner
et al incorporated PS-PEG capped with hydroxyl-groups into PFBT CPNs, and then used the surface hydroxyl groups to conjugate the surface PEG to streptavidin [20]. A recent report uses PEG coupled to DSPE as the encapsulation matrix in miniemulsion CPN syntheses, and again takes advantage of PEG endgroups for covalent attachment (in this case, to targeting TAT peptide) [18].

Other efforts have focused on incorporation of additional (non-PEG) amphiphilic polymers into the CPN core during reprecipitation. Wu et al incorporated polystyrene “decorated” with PEG-like moieties into CPNs and covalently linked the carboxy endgroup to streptavidin, which was subsequently used to tether biotin-linked antibodies for cell-surface receptor labeling [6]. The same group also incorporated poly(styrene-co-maleic anhydride) (PSMA) into reprecipitated CPNs, and then hydrolyzed the maleic anhydride endgroups into carboxy moieties to allow covalent linkage via click chemistry [16]. In theory, incorporation of amphiphiles should increase colloidal stability by reducing surface hydrophobicity. In practice, however, CPNs incorporating amphiphiles may still aggregate in biological buffers and/or require additional surface modification to ensure stability, depending on the choice of synthesis method and amphiphile, as well as the surface amphiphile density. For example, some CPNs incorporating styrene-based amphiphiles were reported to possess long-term stability only after additional protein passivation [6], or require additional polyelectrolyte coating to prevent aggregation in physiological buffer [15]. Hence, while incorporation of amphiphilic molecules allows bioconjugation, it is not a universally effective strategy to generate CPNs that are stable under biological or near-biological conditions. In addition, the majority of methods for
incorporation of designed amphiphilic polymers into the CPN core require in-house polymer synthesis, which makes this strategy unlikely to be generally applied.

In an alternate approach, the surface of otherwise bare CPNs can be coated with molecules that are tethered to the surface by noncovalent interactions. Liu et al. used a folate-conjugated cationic triblock copolymer as an adsorbed coating for polyfluorene CPNs [24]; the polyelectrolyte coating both increased colloidal stability in physiological salt and incorporated folate into the CPN surface to promote cellular uptake via the folate receptor. Similarly, Jin et al demonstrated that CPNs prepared by reprecipitation can be coated with polyelectrolytes containing interspersed regions of hydrophobicity and charge to impede aggregation in physiological buffer and allow bioconjugation [15]. Together, these results demonstrate the effectiveness of adsorbed CPN coatings as a path to colloidal stability and bioconjugation.

We have recently demonstrated that unmodified reprecipitated CPNs are coated by protein when incubated in protein solutions, including serum. Proteins are, by definition, amphiphilic polymers and contain functional groups available for covalent attachment. Adsorbed protein can thus serve the same function(s) as the adsorbed polyelectrolyte or copolymer coatings used by other researchers to improve stability and facilitation bioconjugation. We have demonstrated that a protein corona surrounding PFBT CPNs results in higher colloidal stability and cellular uptake than observed for CPNs without a protein coat. This protein corona is highly stable and does not dissociate from the CPN surface even after removal of excess (solution-phase) protein. In addition, using a surface coating of biotinylated BSA, we have demonstrated that protein-coated
CPNs can be utilized for biorecognition processes. These observations indicate that surface characteristics of protein-coated CPNs are dictated by the protein corona, not the CPN core, consistent with what has been observed for silica, polystyrene, iron oxide, quantum-dot, and other nanoparticles that also acquire a protein-coat in protein solutions [25, 26]. We have also demonstrated that protein-coated CPNs can be formed either by incubation of preformed CPNs with protein solutions or more directly, by incorporation of protein into the CPNs during reprecipitation. Notably, including protein into the aqueous phase during reprecipitation allows straightforward synthesis of protein-functionalized CPNs in a single step. Here we expand this work to demonstrate that incorporation of protein into the NP surface is a flexible and straightforward strategy to create CPNs modified with a series of useful biomolecular recognition reagents, including neutravidin, biotin, immunoglobulin (IgG), and protein-A. We further demonstrate use of these protein-modified CPNs in a series of useful analytical assays.

5.2 MATERIALS AND METHODS

5.2.1 REAGENTS

All chemicals and reagents used were commercially available. Each of polyfluorene conjugated polymer PFBT (poly[9,9-dioctyfluorenyl-2,7-diyl]-co-(1,4-benzo-{2,1’,3’}-thiadiazole)], (M_w 48,000, polydispersity 2.7.) (American Dye Source), neutravidin (Thermo Scientific), polyclonal goat anti-rabbit IgG (GenScript), polyclonal anti rabbit (goat) IgG and biotin conjugated anti rabbit IgG (goat) (Rockland antibodies), bovine albumin serum (BSA) fraction V, (Omnipur), protein-A (Weed Scientific
laboratory), sulfo-NHS -biotin (Thermo scientific) were used without further purification. BSA-biotin was prepared by labeling of biotin following pierce protocol for labeling of proteins. All water used was Barnstead Nanopure deionized water (18.2 MΩ) that had been further filtered through 20 nm PVDF filter (Whatman).

5.2.2 NANOPARTICLE SYNTHESIS

Protein-modified PFBT CPNs were prepared by reprecipitation, as previously described. Briefly, PFBT polymer stock solution was dissolved in tetrahydrofuran (THF) to make a 250 ppm solution that was then rapidly diluted 1:10 in 10 mL phosphate buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 10m M Na₂HPO₄, 1.8 mM KH₂PO₄ at pH 7.4) containing 250 nM protein, under continuous sonication with a micro-tip equipped sonicator (Branson; 45% power, corresponding to 67.5 W). Sonication was continued after dilution, to make a total of 2 minutes sonication. The nanoparticle suspension was filtered through a 220 nm PVDF membrane syringe filter, and nanoparticles were separated from excess protein and THF using size exclusion chromatography using Sephacryal S 200 stationary phase as the stationary phase and phosphate buffered saline as the mobile phase. The molar concentration of 25 ppm PFBT was calculated to be 4.0 nM based on the mass of the conjugated polymer diluted in water during reprecipitation, and the size of the CPN core as measured for PBFT-BSA by TEM. IgG-functionalized PFBT CPNs with a surface coating of IgG were prepared by incubation of Protein-A functionalized CPNs with 250 nM goat IgG in PBS for a period of 24 h, followed by removal of excess IgG by size exclusion chromatography, as above.
5.2.3 NANOPARTICLE SIZE MEASUREMENT

The hydrodynamic size of the CPNs were measured by Photon Correlation Spectroscopy (PCS), also called Dynamic Light Scattering (DLS), using a Malvern Zetasizer (ZS90). DLS experiments were performed in the backscattering mode (scattering angle of 173⁰) at 25⁰C using 1x PBS as dispersant. Refractive index of PFBT in THF at 25 ppm was 1.40 as measured using refractometer (Abbe refractometer, model 2WAJ). Prior to each measurement, CPN samples were sonicated for 20 seconds in a bath sonicator to remove bubbles and minimize possible transitory/unstable aggregates. The Z-average mean hydrodynamic size and polydispersity index were determined and analyzed in terms of intensity weighted distributions using cumulant analysis. Measurements were performed on each of three separate preparations; the mean and standard deviation were calculated for these triplicate data.

5.2.4 SPECTROPHOTOMETRIC MEASUREMENTS

Fluorescence signal was obtained from emission spectra obtained with a steady state spectrofluorometer (Photon Technology International, PTI, Quantmaster), using 460 nm excitation (2 nm band pass) and emission scanned from 480nm- 650nm (1 nm step: 2 nm band pass). Fluorescence associated with the CPNs was quantified by integrating the emission spectra from 500 nm to 600nm. Absorbance spectra were acquired using 10 ppm CPNs in PBS with UV-VIS recording spectrophotometer (UV-2501PC, Shimadzu) in water. Spectra were collected from 350 nm to 600 nm.
5.2.5 SOLID PHASE IMMUNOASSAY

Antigen (rabbit IgG) was coated on 96 well micro plate surface at various concentrations (0.1nM to 200 nM) in phosphate buffer saline (PBS) at pH 7.4 and incubated for two hours at room temperature. After removal of non-adsorbed rabbit IgG and washing 3 times with washing buffer (PBS/0.05% Tween 20), the well surfaces were blocked with 4% non-fat dry milk in PBS/0.05% Tween 2, followed by three washes with washing buffer. For immunoassays using detection with neutravidin-functionalized CPNs, biotinylated goat anti-rabbit IgG (1:100,000 dilution 4mg/ml stock) (200 µL) was added at this point and incubated for two hours in room temperature, followed by three buffer washes; other immunoassays proceeded directly to addition of CPN detection reagent. The detection agent, CPNs coated with relevant protein (goat anti-rabbit IgG, protein-A coupled to goat anti-rabbit IgG, or neutravidin) was added to the final concentration of 0.02 nM in 200 µL PBS and incubated overnight at 4°C, followed by washing 3 times with washing buffer and addition of 200 µL PBS. Fluorescence intensity of CPNs bound to antigen was measured by reading fluorescence in microplate reader (Tecan, GENios) using 485 nm excitation and 535 nm emission (gain 90, and integration time 20 µs). Fluorescence intensity was corrected for background (fluorescence of antigen wells in the absence of CPNs) and converted to binding plots assuming 1:1 binding of nanoparticle to antigen. Data were fit to a single site binding isotherm using nonlinear least squares analysis in SigmaPlot.
5.3 RESULTS AND DISCUSSION

Protein-functionalized CPNs can be prepared in single-step. We previously introduced a straightforward reprecipitation method for synthesis of PEG-lipid coated CPNs, in which conjugated polymer in a small amount of organic solvent is diluted into an aqueous solution of PEG lipid under continuous sonication [3]. As the conjugated polymer precipitates, the lipid portion of the PEG-lipid inserts between portions of the conjugated polymer chain, while the polar PEG extends out into solution. The result is a nanoparticle composed of a conjugated polymer core surrounded by surface corona of PEG. We recently demonstrated that direct substitution of protein for the PEG-Lipid results in CPNs modified with a surface layer of protein. In this case, hydrophobic patches on the protein presumably interact with the hydrophobic conjugated polymer during synthesis and are incorporated into the CPN surface during particle formation, leaving hydrophilic regions on the protein free to extend out into solution. Given the importance of protein-modification for bioconjugation of protein, we asked whether this one-step reprecipitation protocol could be used to prepare CPNs coated with protein moieties useful for bioanalytical or diagnostic assays. We chose test proteins with high utility in a variety of molecular recognition strategies: neutravidin (which can be used to tether commercially available biotin-linked recognition moieties such as nucleic acids, peptides, and antibodies), a representative polyclonal IgG (in this case, goat anti-rabbit IgG); and protein-A (which specifically binds the constant region (Fc) of IgG molecules and can therefore be used as a foundation for tethering any IgG). We also included biotinylated BSA as a test protein; we have previously demonstrated that biotinylated
BSA adsorbed to the CPN surface serves to effectively present biotin for streptavidin recognition but had not previously incorporated biotinylated BSA into CPNs directly during reprecipitation, as carried out here. Our goal was to create a toolbox of protein-coated CPNs that could be used in a wide variety of molecular recognition and detection applications.

To prepare the protein-coated CPNs, PFBT dissolved in a small volume of THF was diluted ten-fold into a solution of the relevant protein in water, under brief (2 min) sonication, as previously described. A recent study of protein corona formation around a variety of nanoparticle types indicates that protein corona formation can be rapid (less than 30 s) [27, 28] and a stable corona is likely to coat the CPN completely during sonication. CPNs formed were immediately removed from both THF and excess (unincorporated) protein was removed via size exclusion chromatography into either PBS or water, depending on the desired application. The result was a suspension of protein-coated CPNs, created in a single step. Notably, this direct method takes dramatically less time than other protocols for synthesis of CPNs adsorbed with amphiphiles, since it requires neither an overnight evaporation to remove THF nor an extended incubation with polyelectrolyte or amphiphiles.

Following CPN synthesis, we used dynamic light scattering to verify nanoparticle production and to determine the respective hydrodynamic diameters of protein-coated particles. Comparison of the size of CPNs prepared using this protocol to that for unmodified (bare) CPNs prepared in the absence of added protein shows significantly increased hydrodynamic radius (Table 1) reflecting the presence of a protein layer.
surrounding the CPN core. Bare (unmodified) PFBT CPNs in water have a hydrodynamic
diameter of 55 nm. However, the hydrodynamic diameters for PFBT CPNs prepared by
dilution into protein solutions ranged from 80 nm (BSA-biotin coated CPNs) to 146 nm
for anti-rabbit IgG-coated CPNs. The relative sizes of the different CPNs are consistent
with the size of the different proteins incorporated at the CPN surface and roughly follow
the relative protein sizes; the largest hydrodynamic diameter is obtained for CPNs coated
with IgG (MW 150,000 kDa) while the smallest hydrodynamic diameter is obtained for
CPNs coated with BSA (MW 66.5 kDa). Size increases do not strictly mirror the sizes of
the free proteins (i.e. increases in CPN diameter are not exactly twice the solution-phase
diameter of the incorporated proteins). A number of studies have suggested that protein
unfolding can occur upon binding to hydrophobic nanoparticles [29-31], presumably to
allow interaction of hydrophobic residues in the protein interior with the nanoparticle
surface. Hence, the diameter of a monolayer of proteins at the CPN surface is expected to
be somewhat different than the diameter of the same proteins in solution.
Table 1. Size Characteristics of Protein-coated PFBT CPNs. Hydrodynamic diameters CPNs were obtained by Z-average cumulant analysis of Light Scattering; measurements of protein-encapsulated CPNs were in PBS); bare particle data were obtained in water to avoid aggregation. Low Polydispersity Indices reflect uniform coating of PFBT Nps; the PFBT polymer itself has some polydispersity, which contributes to observed polydispersity indices. Bare PFBT CPNs prepared under similar conditions have a zeta potential of -36.7 ± 0.9 mV.

<table>
<thead>
<tr>
<th>CPN composition</th>
<th>Hydrodynamic radius (Z-avg) in buffer (nm)</th>
<th>Polydispersity index</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutravidin-PFBT</td>
<td>114 ± 2</td>
<td>0.12</td>
<td>-28.0 ± 0.9</td>
</tr>
<tr>
<td>Anti-rabbit IgG-PFBT</td>
<td>146 ± 3</td>
<td>0.11</td>
<td>-22.4 ± 0.9</td>
</tr>
<tr>
<td>Protein A-PFBT</td>
<td>106 ±1</td>
<td>0.16</td>
<td>-14.2±0.9</td>
</tr>
<tr>
<td>Biotin-BSA-PFBT</td>
<td>80 ± 2</td>
<td>0.12</td>
<td>-26 ± 1</td>
</tr>
</tbody>
</table>

We also measured the zeta potential of both bare and protein-coated CPNs. We have previously reported that bare PFBT Nps have a significantly negative zeta potential, and that a protein corona surrounding CPNs results in less negative zeta potentials (Ackroyd and Kandel, submitted manuscript), consistent with occlusion of conjugated polymer oxidative defects[32,33] by less negatively-charged protein molecules at the CPN surface. As shown in Table 1, direct incorporation of protein at the CPN surface
during reprecipitation reduces the observed zeta potential in a similar and protein-specific fashion. While our uncoated PFBT CPNs have a zeta potential of $-36.7 \pm 0.9$ mV, zeta potentials for protein-coated CPNs ranged from $-28$ to $-12$ mV. In addition, the zeta potential measured for PFBT NPs with a surface coating of biotinylated BSA ($-26 \pm 1$) is indistinguishable from the value previously measured for BSA-coated CPNs ($-26.0 \pm 0.5$ mV) (Ackroyd and Kandel, submitted manuscript). Together, the size and zeta potential measurements confirm formation of a protein coat surrounding PFBT CPNs upon precipitation into protein solution.

5.3.1 COLLOIDAL STABILITY OF PROTEIN COATED CPNs

Protein-coated PFBT CPNs shows colloidal stability in buffer solution. Our CPN reprecipitation protocol results in protein-coated CPNs in water or buffer, without solution-phase protein. As shown in previous work, the size exclusion chromatography step results in removal of essentially all excess (non-adsorbed) protein. Hence, removal of unbound proteins could favor protein dissociation over time. However, in the case that protein-CPN affinity is very high, and/or multi-site interaction results in high avidity, the half-life of bound protein can be sufficiently high that essentially no protein dissociation will be observable, as has been reported for other nanoparticle types [34]. To evaluate whether adsorbed protein was retained on the CPN surface over time, we evaluated the hydrodynamic diameter of the different protein-coated CPNs over a period of 7 days. Figure 1 shows a representative DLS diameter time course over time. For CPNs adsorbed
with IgG, protein-A, or biotinylated BSA, no size change was observed over time (Figure 1), clearly reflecting stable adsorption of these proteins to the CPN surface.

Neutravidin coated particles had stable measured sizes for at least three days (Figure 1). However, measurements at 7 days show approximately two-fold increases in measured size. We conclude that while neutravidin-coated particles are stable for several days, they are best used soon after synthesis. In principle, observed neutravidin-CPN size changes after time could reflect protein dissociation from the CPN surface over time.

![Fig. 1. Colloidal stability of protein-encapsulated CPNs in physiological salt.](image)

Fig. 1. Colloidal stability of protein-encapsulated CPNs in physiological salt. Data reflects intensity-weighted Z-average mean hydrodynamic size of PFBT CPNs functionalized with biotinylated BSA (closed circles), Protein-A (closed triangles), IgG (open circles), and neutravidin (open squares). CPNs were stored and analyzed in PBS. Error bars (largely occluded by symbols) reflect standard deviation of triplicate data.
Alternatively, since neutravidin exists as a tetramer, slow exchange between neutravidin monomers on different nanoparticles could result in gradual aggregation. To determine whether neutravidin dissociation from the CPN surface is occurring, we observed the DLS size of neutravidin-coated particles stored in water rather than PBS. Since bare particles do not aggregate in water over this time period, any dissociation of neutravidin from the CPN surface would be observable as a measured decrease in observed DLS size. We note that the hydrophobic driving force for protein adsorption is higher in salt solutions than in water, so that any protein dissociation from the CPN would be more pronounced in water than in buffer. We observe no change in the DLS size of neutravidin-coated CPNs in water after 7 day incubation (data not shown). We conclude that appreciable neutravidin dissociation from the CPN surface does not occur, and that change in the size of neutravidin-coated CPNs in buffer at 7 days likely reflects neutravidin-neutravidin interaction(s) rather than protein dissociation from the CPN surface.

Together, these DLS time-course data indicate that adsorption of each of IgG, protein-A, biotinylated BSA or neutravidin to the PFBT core is highly stable; protein adsorption under these conditions is “essentially irreversible” [34] and protein adsorption to the CPN core can be considered part of a highly stable “hard corona”. Stable protein adsorption is perhaps not surprising, given the high surface hydrophobicity of PFBT CPNs. Other studies of protein interaction with nanoparticles have demonstrated that protein adsorption increases as the surface hydrophobicity increases [35, 36] and that
protein affinity for hydrophobic nanoparticles is greater than protein affinity for hydrophilic nanoparticles [37].

Table 2. Spectral properties of protein-coated CPNs. For comparison with bare CPNs, which aggregate in PBS, all data were obtained in water using fluorescein as the reference fluorophore. Emission maxima of protein-functionalized PFBT CPNs were indistinguishable from bare.

<table>
<thead>
<tr>
<th>CPN composition</th>
<th>ABSORBANCE MAXIMUM (nm)</th>
<th>QUANTUM YIELD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFBT in water</td>
<td>460.0</td>
<td>13.7± 0.1</td>
</tr>
<tr>
<td>Neutravidin-PFBT</td>
<td>459</td>
<td>13.2± 0.1</td>
</tr>
<tr>
<td>Anti-rabbit IgG-PFBT</td>
<td>459.4</td>
<td>15.1± 0.1</td>
</tr>
<tr>
<td>Protein A-PFBT</td>
<td>461.6</td>
<td>14.7 ± 0.1</td>
</tr>
<tr>
<td>Biotin-BSA-PFBT</td>
<td>459.2</td>
<td>15.3 ± 0.1</td>
</tr>
</tbody>
</table>

5.3.2 SPECTRAL PROPERTIES OF PROTEIN- COATED PFBT CPNs

If protein-functionalized CPNs are to be effectively used as detection reagents for biorecognition, the protein coating must not substantially diminish CPN fluorescent brightness. We investigated the quantum yield of each of protein-A, IgG, and BSA-biotin encapsulated CPNs, and compared them to bare particles. As shown in Table 2, these protein-encapsulated CPNs largely retain the spectral properties of unmodified CPNs, consistent with our previous observations of CPNs with other protein coronas. We have
previously observed that PEG-Lipid coated CPNs show significant increases in quantum yield relative bare CPNs, and have interpreted that observation as reflecting insertion of lipid in between polymer chains in the CPN core, a process that alleviates fluorescent quenching caused by tight chromophore packing [3]. Data in Table 2 suggests that insertion of hydrophobic residues into the CPN core may be occurring; however, any impact on observed quantum yield or absorbance maxima is small and protein-dependent.

5.3.3 PROTEIN-A CPNs AS SCAFFOLDS FOR CPN LINKAGE TO IgG

We have prepared PFBT CPNs coated with protein-A as a general scaffold for bioconjugation to a series of IgG molecules. Protein-A binds the constant region of IgG molecules, well away from the antigen binding site, and should therefore capture IgG molecules and present them in an orientation that allows efficient antigen binding. As proof of principle for IgG attachment to protein-functionalized CPNs, we incubated protein-coated CPNs with polyclonal IgG (in this case, goat anti-rabbit IgG). While the DLS diameter of protein-A PFBT CPNs was 106 ± 1 nm, the same CPNs incubated with IgG had a DLS diameter of 158 ± 3 nm, reflecting tethering of a layer of IgG to the protein-A CPN surface. Similarly, when protein-A coated CPNs were incubated with IgG, their zeta potential changed from -14.7 ± 0.1 to -12.0 ± 0.4 mV, consistent with significant alteration of the CPN surface. No size increases were observed when protein-A coated CPNs were incubated with BSA. We conclude that protein A adsorbed on the CPN surface specifically binds IgG.
To demonstrate the utility of the resulting IgG-linked Protein-A CPNs for application(s) in immunoassays, we carried out a solid phase sandwich immunoassay in which various concentrations of antigen (polyclonal rabbit polyclonal IgG, in this case) were adsorbed on 96-well plates and detected using IgG tethered to protein-A CPNs, as described above. Representative data are shown in Figure 2. Consistent with antigen-antibody interaction, bound CPN fluorescence changes as a function of antigen concentration, and the resulting data can be fit by a single site binding isotherm to yield a $K_d$ of $5.4 \pm 0.7$ nM. Plate immunoassays generally require enzyme-linked amplification
to allow readout. In this case, very high CPN fluorescence brightness allows detection of antigen over a large concentration range without any requirement for signal amplification.

We designed the assay in Figure 2 to reflect antibody-antigen interactions (i.e. goat anti-rabbit IgG binding to antigen), and not protein-A interaction with antigen rabbit IgG. However, since both partners of the antibody-antigen pair we have chosen are IgG in this case, it is theoretically possible that observed CPN fluorescence in the

**Fig. 2 Solid phase immunoassay with detection by Protein-A functionalized CPNs.** Antigen (rabbit IgG) adsorbed to 96-well plates was detected by goat anti-rabbit IgG tethered to protein-A coated PFBT CPNs. Resulting CPN fluorescence data were fit to the single site binding isotherm.
immunoassay could reflect protein-A interaction with antigen IgG rather than primary IgG interaction with antigen IgG. Relatively large (52 nm) increases in the size of Protein-A coated CPNs upon exposure to IgG suggests that any uncapped Protein-A is likely hidden behind a surface layer of IgG, and is sterically inaccessible to antigen. In addition, we observe close agreement between Kd values in immunoassays that use a PFBT-protein-A-IgG sandwich and those using PFBT directly adsorbed with IgG (see below), consistent with binding of antigen rabbit IgG by anti-rabbit IgG, rather than protein-A.

5.3.4 IgG ADSORBED CPNs AS DETECTION AGENT IN IMMUNOASSAY(S)

While we have demonstrated that protein-A CPNs can be used to tether IgG for immunoassays, it would be advantageous to use IgG-coated CPNs without requirement for a mediating protein-A layer, since the resulting particles could be formed in a single synthetic step. However, direct IgG adsorption has the potential to occlude antigen-binding region of the antibodies if the IgG complementarity determining region interacts with the CPN surface. The result could be an IgG-coated CPN that, while stable, would not appreciably bind antigen. To investigate the antigen-binding effectiveness of IgG-coated CPNs prepared by reprecipitation of PFBT into IgG solutions, we incubated CPNs modified with goat anti-rabbit IgG with antigen (in this case, rabbit IgG). The DLS diameter of the IgG-coated PFBT CPNs was 146 ± 2 nm, while the diameter after incubation with antigen was 163 ± 2 nm, reflecting antigen binding by IgG on the CPN
surface. Hence, even after adsorption/incorporation into the CPN surface, the IgG molecules retain the ability to bind antigen. We cannot rule out the possibility that some of the adsorbed IgG is positioned on the CPN surface in a way that impedes antigen binding, since a variety of antibody orientations (and conformations) on the CPN surface are possible. However, the DLS and solid-phase immunoassay demonstrate that a sufficient quantity of IgG on the CPN surface is oriented to allow stable antigen binding.

To further demonstrate that IgG-coated CPNs bind target antigen, and can be used as a specific detection reagent in bioanalytical assays, we carried out additional solid phase immunoassays. As for the previous assay, different concentrations of antigen

![Graph](image)

**Fig. 3. Solid phase immunoassay with detection by IgG-adsorbed CPNs.** Antigen (rabbit IgG) adsorbed to 96-well plates was detected by PFBT CPNs functionalized with anti-rabbit IgG. Resulting CPN fluorescence data were fit to the single site binding isotherm.

To further demonstrate that IgG-coated CPNs bind target antigen, and can be used as a specific detection reagent in bioanalytical assays, we carried out additional solid phase immunoassays. As for the previous assay, different concentrations of antigen
(rabbit IgG) were adsorbed onto 96-well plates using IgG-coated CPNs. In this case, bound antigen was detected with PFBT CPNs coated with goat anti-rabbit IgG. The resulting sigmoidal binding curve is shown in Figure 3. A fit of the single site binding isotherm to this data yields an apparent antibody-antigen $K_d$ of $5.5 \pm 0.8$ nM, a value indistinguishable from that obtained in Figure 1, which used IgG tethered to protein A–coated CPNs as the detection reagent. Agreement between $K_d$ values obtained from these different methods of coating CPNs with IgG suggests that IgG directly adsorbed to the nanoparticle surface binds antigen as effectively as IgG tethered to CPNs via protein-A.

Neutravidin-coated CPNs serve as a scaffold for bioconjugation to biotin-linked recognition moieties. In principle, neutravidin-coated CPNs can serve as a scaffold for attachment of any biotin-linked recognition moiety. Since biotin-linked nucleic acids, peptides, and antibodies are commonly available, neutravidin PFBT CPNs can readily serve as highly bright labels for any diagnostic or cellular imaging sandwich assay that uses these recognition moieties. To demonstrate that a neutravidin corona on PFBT CPNs can be used to tether biotinylated reagents, we incubated neutravidin-coated PFBT CPNs prepared by reprecipitation together with biotinylated goat anti-rabbit antibody. As a control, we also incubated the neutravidin-PFBT CPNs with BSA. The DLS hydrodynamic diameter of the neutravidin antibodies increased when incubated in biotinylated antibody from $114 \pm 2$ nm to $162 \pm 2$ nm, reflecting a surface antibody layer mediated via the biotin-streptavidin interaction; no size increase was observed for the same nanoparticles incubated in BSA ($111 \pm 2$ nm versus $114 \pm 2$ nm in the presence of BSA).
As a demonstration of the utility of these neutravidin-coated CPNs as a highly bright detection label, we carried out a sandwich immunoassay in which antigen binding by biotinylated antibody was detected using freshly synthesized neutravidin-PFBT CPNs. Various concentration of a primary antibody (rabbit IgG) were adsorbed to a 96-well plate. A complementary biotinylated secondary antibody (biotinylated goat anti-rabbit IgG) was used to bind adsorbed primary antibody, and then detected via fluorescence associated with the neutravidin CPNs. The resulting sigmoidal binding curve (Figure 4) fits a single site binding isotherm, yielding IgG-antigen $K_d$ of $4.5 \pm 0.7$ nM, a value indistinguishable from that obtained from previous plate assays using the same antigen-antibody pair, but detected using CPNs with different protein coats.
Adsorbed protein layer is retained under serum conditions. Vroman described a picture of protein interaction with manmade materials in which an initial protein layer is defined by binding kinetics and populated with proteins with high on-rates, although not necessarily high equilibrium affinities [38]. This initial layer would then change over time as low affinity proteins with high on-rates were displaced by higher affinity proteins with slower on-rates [28, 39]. A similar process could operate for nanoparticles; a protein coat surrounding a nanoparticle could be altered by the presence of competing proteins in solution, with the result that any functionalization offered by a protein corona would be undermined in serum or intracellular solutions [26]. To determine whether CPNs
functionalized with proteins would be compromised by the competition with serum proteins, we compared plate immunoassays carried out in the presence of fetal bovine serum. Using a protocol analogous to that used to obtain Figure 2, various concentrations of antigen (rabbit IgG) were detected by measurement of CPN fluorescence after overnight incubation with IgG-functionalized CPNs in 10% FBS. Under these conditions, CPN would bind antigen only in the case that the relevant protein corona was not displaced by serum proteins. As shown in Figure 5, IgG-functionalized CPNs bind target antigen even after overnight incubation with competing serum proteins. Analysis of the resulting binding curve yields an IgG-antigen dissociation constant of 2.1 ± 0.5 nM. We conclude that protein corona-functionalization of CPNs is sufficiently long lived to allow biorecognition in the presence of competing proteins.

Fig. 5. Solid phase immunoassay with CPN detection in the presence of serum. Antigen (rabbit IgG) adsorbed to 96-well plates was detected by goat anti-rabbit IgG tethered to protein-A coated PFBT CPNs in the presence of 10% FBS. Resulting CPN fluorescence data were fit to the single site binding isotherm.
5.3.5 DISCUSSION

Data provided here demonstrates that direct protein adsorption during synthesis allows straightforward protein encapsulation of CPNs. If the CPNs are formed by reprecipitation into protein solutions and excess protein and residual solvent are immediately removed by size exclusion, as performed here, then protein-functionalized CPNs can be synthesized quickly, in a few hours, without requirement for overnight THF removal or extended incubation in protein solutions. This protein coating both improves colloidal stability under physiological salt conditions and allows CPNs to be functionalized with proteins of biological interest. We demonstrate modification of PFBT CPNs with relevant recognition proteins, including protein-A, immunoglobulin, neutravidin, and biotinylated BSA. Together, this collection of CPN surface modification should allow easy adaptation of ultrabright CPNs as detection reagents for a range of useful bioassays. Hydrophobic CPNs have not, in general, been utilized as detection reagents for \textit{in vitro} diagnostic assays, with limited proof of principle exceptions [7, 40, 41]. However, this easy protein functionalization has the potential to greatly expand CPN utility, provided that the protein corona is both stable and available for biorecognition.

Since the protein coronas formed here are not covalently linked to the CPN surface, one potential criticism is that the protein corona could dissociate from the CPN surface over time. The result would be loss of both functionality and colloidal stability in buffer, with resulting loss of overall utility. Our observations of DLS size after extended incubation in protein-free solutions indicate that dissociation of the tested coronas does not occur at an observable rate, either because the hydrophobic adsorption is sufficiently
stable, or because multisite interaction (avidity) ensures that single-site dissociation is followed rapidly by re-association. Hence, the relevant protein coronas are not readily removed from the CPN core; protein functionalization via adsorption is highly stable.

Several protein corona studies have indicated that protein adsorption can result in partial protein unfolding and/or denaturation [29-31, 42]. Hence, a stable adsorbed protein coat may not be able to perform its relevant endogenous function, since adsorption could alter conformation sufficiently to preclude biomolecular recognition. We have not carried out a systematic study of the degree of protein unfolding caused by CPN binding. However, our investigations of the binding activity of CPNs functionalized with IgG, protein-A, and neutravidin indicates that when directly adsorbed to PFBT CPNs, these proteins form stable complexes with their relevant binding partners. While protein adsorption to CPNs could involve a variety of protein orientation and conformations, some of which could be sterically or conformationally inappropriate for molecular recognition, our DLS and immunoassay data shows that a substantial portion of the protein corona is both accessible and sufficiently folded for binding. Indeed, apparent $K_d$s obtained from IgGs tethered to CPNs via protein-A was identical to that from IgGs directly adsorbed to the CPN surface. In this case at least, direct adsorption has no apparent impact on binding affinity.

An additional potential concern is that relevant protein functionalization could be displaced by competing proteins present in biological solutions (e.g. in serum or under intracellular or intra-organellar conditions) [26, 43]. In that case, formation of a stable corona in protein-free solutions does not imply stable protein functionalization under
individual assay- or cellular imaging conditions. To investigate whether a preformed protein corona can survive in the presence of competing proteins, we compared results of plate immunoassays in the presence and absence of serum. We obtained sigmoidal binding curves under both conditions, with mildly higher affinity binding in the presence of serum presumably reflecting tighter binding under increased osmotic stress. While we cannot rule out the possibility that serum proteins could have displaced some portion of the protein-A corona during the course of the overnight incubation with serum, it is clear that sufficient protein functionalization remains for biorecognition; these protein-encapsulated CPN can be used as detection reagents in the presence of competing protein(s), such as those present in serum or inside cells.

Recent modeling of nanoparticle protein corona formation argues that in mixed protein solutions, an initial metastable corona of a specific composition will change over time to a more stable composition [28, 39]. Since we have chosen our protein coronas for practical utility rather than known highest equilibrium affinity for the CPN surface, this model suggests that displacement of the preformed corona by higher affinity plasma or serum proteins could be a significant experimental complication, particularly for experiments requiring long incubations. However, our protein corona functionalization varies from the model in that the functionalized CPNs start with a preformed and stable protein corona (a “hard corona”) before exposure to the mixed protein solution. Studies of the protein composition of the corona surrounding different nanoparticle types over time in complex protein mixtures indicates that while the percentage composition of corona proteins can change over time [43], so does the density of proteins in the
Importantly, after a relatively dense protein monolayer is achieved, few overall changes in protein composition occur [34]. While the concurrence of a dense associated protein layer and minimal changes in composition has typically been interpreted as reflecting binding of proteins with the highest equilibrium binding affinity [26], an alternate explanation is that a dense existing protein coat causes competing solution-phase proteins to be largely excluded from proximity to the CPN surface, much as a high density large MW PEG coat excludes competing proteins. Hence, in the presence of a dense protein layer, the avidity of protein adsorption could cause re-association of a given protein with the CPN surface before any solution phase proteins could approach closely enough to exchange into the hard corona, regardless of the relative affinities of the different proteins. We hypothesize that incorporation of protein into the CPN core during formation (i.e. a performed hard corona formed during CPN synthesis) promotes a sufficiently high protein density that solution phase proteins are excluded from the CPN surface, with the result that displacement by competing proteins is minimized. Other groups have reported that for individual nanoparticle types, a preformed corona was not displaced by serum proteins [27, 46], and that a preformed corona was protective against competing protein adsorption [47].

5.4 CONCLUSION

The easy methodology for production of highly stable protein-functionalized CPNs reported here allows flexible application of CPNs as detection reagents for virtually any in vitro molecular recognition assay. For example, IgG-functionalized CPNs
(formed either by direct IgG adsorption or tethered to Protein-A adsorbed on the CPN surface) can be used as detection reagents in a range of immunoassays, as demonstrated here. Depending on the choice of IgG, these functionalized CPNs could also be used as specific labels in western blotting, fixed cell imaging, or diagnostic assays. Neutravidin-functionalized CPNs are a flexible foundation for tethering the range of available biotinylated reagents to the CPN surface. In all of these applications, the high inherent brightness of the CPNs would be a huge advantage. It is our expectation that straightforward and relevant protein functionalization, as demonstrated here, will greatly increase the widespread use of CPNs as fluorescent labels for sensitive detection in bioassays.

5.5 REFERENCES


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CHAPTER SIX
GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The impetus for studying conjugated polymer nanoparticles (CPNs) comes from their high fluorescence brightness and reasonable photostability. As such, CPNs find applications in cell labeling, sensing, and long term imaging. However, such applications are hindered by the inertness of the CPN surface as well as the limited colloidal stability of CPNs in biological media. The research presented in this thesis developed and characterized several methods of surface passivation to functionalize CPN surface and maintain colloidal stability in high ionic strength solution.

Here, we prepared extremely bright, functionalized and stable conjugated polymer nanoparticles by the inclusion of end group functionalized PEG-Lipids. We demonstrated that functionalized nanoparticles exhibit improved optical properties, long term solution stability and can be used for targeted cellular labeling of cell surface markers. In another study, we compared the fluorescence brightness of cells loaded with PEG-Lipid modified CPNs to cells loaded with quantum dots (Qdots) and dextran linked small molecule organic dye of similar sizes. Our results showed that CPNs are 175x brighter than Q dots and 1400x brighter than Alexa fluor when evaluated in flow cytometry. Our data suggests that extreme brightness of CPNs allow the use of extremely low loading concentration indicating CPNs are appropriate and attractive candidates as fluid phase markers.

In a subsequent chapter, we characterized and tested methods of protein adsorption on the CPN surface to provide colloidal stability and to allow bioconjugation.
Unmodified hydrophobic CPNs tend to aggregate in saline solution and biological media with high salt concentration. We found that incubation of CPNs with proteins such as bovine albumin serum, lysozyme, and fetal bovine serum created a strongly associated CPN surface coating. Such a coating not only provides colloidal stability in high ionic strength solution over a wide pH range, but also functionality for bioconjugation. Similarly, we demonstrated that a formation of stable PFBT CPN corona occurred with immunoglobulin, protein-A and neutravidin. We then utilized the protein functionalized CPNs as detection reagents in solid phase immunoassays. Taken together, we conclude that surface passivation of CPN with functional phospholipid and protein offers a straightforward functionalization method for CPNs. This in turn enables CPNs to be widespread fluorescent label in biological imaging and sensitive detection in bioassays.

However, to realize the widespread use of CPNs in biological applications, some issues remain. A consistent theme in this thesis was on the study of the methods to introduce functionality on the CPNs surface by incorporation of functional polymers and proteins. Surface decoration of CPN with different proteins and targeting ligands results in multifunctional nanoparticles which has great potential for imaging and delivery of biologically relevant molecules simultaneously. So, nanoparticle formulation and surface decoration with various proteins and targeting ligands comprise future work. As shown in chapter 3, PEG lipid modified CPNs can be used as bright fluid phase markers in macrophage cells. Targeted intracellular labeling improves sensitivity and limit of detection. Further studies examining the mechanism of cellular uptake and intracellular distribution of functionalized CPNs are required to gain insights about efficiency of
intracellular labeling. In addition, future work includes the stability and efficacy of decorated CPNs in intracellular environment. This work presented in this thesis provides the basis on which the proposed studies can be built. Altogether, these studies will provide important information concerning the formulation of CPN for biological imaging sensing and detection.