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Mechanism of Cellular Uptake of Highly Fluorescent Conjugated Polymer Nanoparticles

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Abstract

Conjugated polymer nanoparticles are formed by precipitation of highly fluorescent conjugated polymers to form small nanoparticles with extremely bright fluorescence. We characterized cellular uptake and cytotoxicity of 18 ± 5 nm PFBT conjugated polymer nanoparticles in J774A.1 cells. Significant nanoparticle uptake was observed, indicating efficient nanoparticle entry into cells, even for short (1 h) incubations. The high fluorescence of these nanoparticles allows extremely low loading concentrations; PFBT nanoparticle fluorescence in cells could be detected with loading concentrations of 155 pM (270 ppb). Cellular uptake slows at low temperature, consistent with endocytic entry. Nanoparticles colocalize with Texas Red dextran and are trafficked to lysosomes, as demonstrated by the location of nanoparticle fluorescence in perinuclear organelles that also stain with an anti-LAMP-1 antibody. Inhibition of uptake by phosphoinositide 3-kinase inhibitors implicates macropinocytosis as the operative endocytic mechanism. No significant cytotoxic or inflammatory effects could be observed, making PFBT nanoparticles attractive probes for live cell imaging.

Keywords

Intracellular; imaging; cytotoxicity; macropinocytosis; fluorescence; probe

INTRODUCTION

The use of fluorescent reporters in live cell imaging is both widespread and has a long history. By targeting dye molecules to specific locations within the cell, significant improvements in image contrast are achieved for the labeled organelles or cellular structures compared to transmitted light microscopy. For example, the small molecule polar tracer fluorescein is taken up by living cells into endocytic compartments. Unfortunately, while the fluorescence from labeled organelles is observable when loaded with micromolar fluorophore concentrations, image contrast is rapidly degraded due to the tendency to photobleach and susceptibility to rapid clearance from the cell¹ via cellular efflux pathways (e.g. organic anion transporters² when not conjugated to macromolecules such as dextrans or proteins. In addition, most traditional organic fluorophores have limited utility for single molecule imaging in cells.

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SUPPORTING INFORMATION AVAILABLE: Supporting Information is available and contains supplementary experimental details and figures not included in the main article. These figures include absorbance and fluorescence spectra of CP nanoparticles (Figure S1), cell viability controls (Figure S2), nanoparticle uptake by CHO-K1 cells (Figure S3), colocalization experiments for CP nanoparticles with clathrin heavy chain (Figure S4) and caveolin-1 (Figure S6), and actin filament distribution (Figure S6). This material is available free of charge via the Internet at <http://pubs.acs.org>.

An array of nanomaterials, including colloidal inorganic semiconductor quantum dots^{3, 4}, dye-doped silica colloids⁵, dye-loaded latex or polystyrene nanospheres⁶, and fluorescent noble metal nanoparticles and nanoclusters⁷ have been used as alternatives to traditional fluorophores. Nanoparticles often exhibit higher fluorescence, higher photostability, and lower susceptibility to cellular efflux mechanisms than small molecule labels⁸. In addition, surface functionalization of these nanomaterials can promote solubility and/or facilitated or active delivery to specific cellular targets^{3, 5, 9, 10}. Nanoparticles have been widely used in biological imaging, including applications ranging from fixed and live cell imaging to deep tissue and vascular imaging^{11, 12}. However, existing nanoparticles have been criticized for their potential for cytotoxicity¹³, which can result from either the nanoparticle or shell composition^{12, 14, 15}. Cytotoxicity is a particular concern for semiconductor quantum dots, which may leach heavy metals^{16, 17}.

Recently, extremely bright conjugated polymer (CP) nanoparticles have been described¹⁸. Termed “polymer dots” or “CP-dots”, these nanoparticles are formed by reprecipitation^{19–22} of highly fluorescent conjugated polymers. When rapidly diluted from organic solvent into water, the polymer molecules collapse to create nanoparticles by exclusion of water from the hydrophobic interior of the particle. The extremely bright fluorescence of CP nanoparticles is a result of their high absorption cross sections and quantum yields as high as 40%²¹, yielding fluorescence signals that greatly exceed small organic fluorophores and other nanoparticles of similar size. For example, 15 nm PFPV (poly[(9,9-dioctyl-2,7-divinylene-fluorenylene)-alt-co-{2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene}]) nanoparticles have a measured absorbance cross section of $5.5 \times 10^{-13} \text{ cm}^2$ ^{20, 21}, a value 10-fold greater than quantum dot nanoparticles and 1000-fold greater than typical small molecule dyes. In addition, these polymer nanoparticles can be constructed from a blend of two different polymers²³ or doped with specific dyes^{21, 24} to tune nanoparticle excitation and emission characteristics. CP nanoparticles exhibit excellent figures of merit for multicolor applications²¹, two-photon imaging²⁵, oxygen sensing²⁶, and single particle tracking²⁷. Larger (50–250 nm) CP nanospheres referred to as semiconducting polymer nanospheres have also been prepared using an alternative miniemulsion process^{28, 29}. Preliminary investigations have been made on their potential cellular uptake mechanisms and cytotoxicity^{30–32}.

While CP nanoparticles show significant potential for application as biological probes at low concentrations, their specific effects on cells have not been investigated in depth. In this study we explore the suitability of these reprecipitated CP nanoparticles for application in live cell imaging and other cell based assays. Using poly[(9,9-dioctylfluorenyl-2,7-diyl)-co-(1,4-benzo-{2,1',3'}-thiadazole)] (PFBT) nanoparticles as representative CP nanoparticles, we have examined the cellular uptake, cytotoxicity and inflammatory effects of nanoparticle uptake in the mouse macrophage-like J774A.1 cell line. The resulting data indicate that in addition to their bright fluorescence, these CP nanoparticles are localized to specific organelles in the cell and show no significant cytotoxicity at our working concentrations, making them attractive candidates for use in biological imaging.

EXPERIMENTAL

Reagents

The J774A.1 mouse macrophage-like cell line was obtained from American Type Culture Collections. Texas red dextran (TR-dex; $M_r = 10,000$ MW), TRIZOL reagent, and SuperScript First Strand synthesis system for RT-PCR were obtained from Invitrogen. The conjugated polymer PFBT ($M_r = 10,000$; Polydispersity = 1.7) was purchased from American Dye Source (Quebec, Canada). PCR primers were synthesized by Integrated DNA Technologies. GoTaq Master Mix and Cell Titer Blue were purchased from Promega.

Interferon gamma and LPS were purchased from Fisher Scientific and Sigma-Aldrich respectively. Monoclonal antibodies against Clathrin Heavy Chain (D36C) and Caveolin-1 (D46G3) were obtained from Cell Signaling Technology as well as goat anti-rabbit IgG F(ab')₂ fragment conjugated with Alexa Fluor 647 as a secondary antibody. The rat anti-mouse LAMP-1 antibody conjugated with allophycocyanin (APC) was purchased from Southern Biotechnology. All other chemical reagents used in this study were obtained from Fisher Scientific.

Nanoparticle preparation and characterization

Conjugated polymer nanoparticle preparation was carried out as described previously²⁷. Briefly, 20 mg of the conjugated polymer PFBT was dissolved in 10 g of HPLC-grade tetrahydrofuran (THF) by stirring overnight under inert atmosphere. The solution was then filtered through a 0.7 micron glass fiber filter in order to remove any insoluble material. Then 400 μ L of the solution above (2000 ppm) was injected by pipette into 8 mL water under mild sonication using a commercial ultrasonic bath (Bransonic) for 1 minute. This solution was then heated in a water bath (~70°C) under constant N₂-bubbling until THF and half of the water was removed. Next, a second batch (400 μ L into 8 mL) of a prepared nanoparticle solution was added, mixed with the previously concentrated solution and heated and under constant N₂ bubbling for another time period to remove THF. 5 mL of the resulting nanoparticle solution remained after THF removal and this solution was dialyzed against 0.01 M borate buffer (pH = 8.5) twice for 12 h. Finally, 5.5 mL PFBT nanoparticle solution in 0.01 M borate was obtained.

Nanoparticles were characterized by atomic force microscopy (AFM); PFBT nanoparticle solutions were dried on mica or prepared glass cover slips and height images were recorded as described previously²¹. AFM size distributions were determined from these images to be 18 ± 5 nm.

Nanoparticle concentrations were estimated from the mass of conjugated polymer starting material diluted into aqueous solution and the volume of individual particles, assuming complete polymer to nanoparticle conversion. Specifically, particle heights measured by AFM were used to calculate nanoparticle volume, assuming a spherical shape. Nanoparticle volumes were converted to single nanoparticle mass, assuming a nanoparticle density of 1 g/cm³ (actual density is between 0.95 and 1.05 g/cm³). The total mass of conjugated polymer diluted in the reprecipitation, divided by the mass of a single nanoparticle, yields the number of nanoparticles formed, and is easily converted to moles of nanoparticles. Moles of nanoparticles divided by the final volume of the preparation solution yields the final molar concentration of nanoparticles. Concentration calculations do not take into account small ($\leq 3\%$) reductions in yield that result from filtration (UV-vis measurements are made before and after filtration) and may therefore be a slight overestimate. Based on these calculations the nanoparticle concentration was 250 ppm/140 nM in 0.01 M borate buffer. From the calculated concentration and the measured absorbance, an absorbance cross section was calculated for the PFBT nanoparticles in water (1.8×10^{-13} cm²), consistent with previously reported values²¹. The maximum volume of PFBT nanoparticles added to cells in these studies was 10% of the total culture volume to minimize any effects of the vehicle.

Cell Culture

The J774A.1 mouse macrophage-like cell line was obtained from American Type Culture Collections and grown in high glucose Dulbecco's Modified Eagle Medium (DMEM; Mediatech) supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone), penicillin/streptomycin, sodium pyruvate, and L-glutamine at 37°C in a humidified incubator with 5% CO₂ until reaching 80–90% confluence. Cells were propagated by

incubating for ≥ 5 minutes in ice cold phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.2) followed by gentle pipetting then split in a 1:4 to 1:8 ratio.

Flow cytometry

Cells were analyzed using flow cytometry by first resuspending them in ice cold Ringer's buffer (RB; 155 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 2 mM NaH_2PO_4 , 10 mM glucose, 10 mM HEPES, pH 7.2–7.4) with gentle pipetting and were analyzed on a Becton-Dickenson FACScan flow cytometer. For all experiments, excitation utilized the 488 nm line from a 15 mW argon ion laser and the green fluorescence channel (FL1). For each sample, 10,000 cells were measured. Data was analyzed using FlowJo software (Treestar).

Fluorescence microscopy

All fluorescence images were acquired with an inverted fluorescence microscope (Olympus IX71) equipped with fluorescence excitation from a 300W Xe arc lamp coupled to the microscope via a liquid light guide (Sutter Instruments). Differential interference contrast (DIC) was used for transmitted light imaging. The microscope was equipped with both excitation and emission filter wheels (Sutter Instruments). A Sedat set consisting of a beam splitter, single band excitation filters [387 nm (11 nm band pass), 494 nm (20 nm band pass), 575 nm (25 nm band pass)], and single band emission filters [447 nm (60 nm band pass), 531 nm (22 nm band pass), 624 nm (40 nm band pass)] (Semrock) was used for all experiments. Finally, an Orca-ER CCD (Hamamatsu) was used for image acquisition. Control of all microscope components and all image processing was performed using Slidebook 5.0 (Intelligent Imaging Innovations).

Nanoparticle uptake

Cells were grown in 35 mm tissue culture dishes until $\sim 70\%$ confluent, then treated with PFBT nanoparticles. The maximum final concentration of nanoparticles was 10-fold less than the PFBT nanoparticle stock solution to avoid more than 10% dilution of media. Cells were washed 3X in ice cold RB and detached via gentle pipetting and analyzed by flow cytometry. For imaging of nanoparticle uptake, cells were plated at 100K/dish in glass bottom dishes and incubated with PFBT nanoparticles. Cells were washed 3X in RB and the optical bottom dishes were placed on the microscope in a microscope stage heater at 37°C (Warner Instruments) and images acquired.

Colocalization with TR-dex

J774A.1 cells were plated at 100K/dish in glass bottom dishes and incubated with 2 nM (4 ppm) PFBT nanoparticles and 250 nM TR-dex overnight. Cells were washed 3X in RB and the optical bottom dishes were placed on the microscope in a microscope stage heater at 37°C and images acquired.

Immunofluorescence and colocalization

The cells were plated at 100K/dish in glass bottom dishes and incubated with 2 nM (4 ppm) PFBT nanoparticles from 2–16 hours. Cells were washed in DMEM and chased from 0–4 hours. Following labeling with nanoparticles, the cells were fixed at 37°C with 4% paraformaldehyde in RB for 10 minutes then washed 3X in RB. The fixed cells were incubated in blocking buffer (BB; RB + 1.5% v/v bovine serum albumin (BSA) and 0.3% Triton-X) for 2 h at 4°C . Next, cells were incubated with the 1° antibody in RB + 1% BSA and 0.3% Triton-X for 16 h at 4°C . Dilutions of 1° antibodies were as follows: LAMP-1 (1:200); clathrin heavy chain (1:200); caveolin-1 (1:200). Following incubation with the 1° antibodies, cells were washed 3×15 min in BB at room temperature and incubated with the

Alexa fluor 647 2° conjugate (1:1000) for 2 h at room temperature in RB + 1% BSA and 0.3% Triton-X. Finally, the cells were washed 3 × 15 min in BB and images acquired.

Blocking nanoparticle uptake using inhibitors of endocytosis

Known inhibitors of various endocytic processes were used to help elucidate the mechanism of cellular uptake of CP nanoparticles. Cells were plated in 35 mm tissue culture plates and grown to ~ 70% confluence. The cells were preincubated with various inhibitors: methyl- β -cyclodextrin (2.5 mg/ml), wortmannin (100 ng/ml), LY294002 (20 μ g/ml), cytochalasin D (10 μ g/ml), nocodazole (10 μ g/ml), genistein (10 μ g/ml), and chlorpromazine (1 μ g/ml) for 30 min followed by addition of 2 nM (4 ppm) PFBT nanoparticles for 1.5–2 h. Cells were then washed 3X in ice cold PBS and resuspended by gentle pipetting. Cells were analyzed by flow cytometry. Statistical analysis of the mean fluorescence for each treatment compared to the untreated and vehicle controls was done using ANOVA in Sigmaplot. An inhibitor cytotoxicity control was run using propidium iodide staining to insure that there was less than 10% cytotoxicity from the inhibitor alone and concentrations were adjusted as needed.

Cytotoxicity of PFBT nanoparticles

The Cell Titer Blue assay was used to assess cytotoxicity of PFBT nanoparticles. Cells were plated at 10K/well in a black 96-well plate and incubated with indicated concentrations of PFBT nanoparticles for 16 h. Cell Titer Blue reagent was added according to manufacturer instructions and incubated with the cells for an additional 2 h then the plate was read out using a fluorescence plate reader (Tecan). Statistical analysis of cell viability versus the untreated control was done using ANOVA in Sigmaplot

RT-PCR of inflammatory markers

J774A.1 cells were grown in 35mm tissue culture plates. When the cells were ~ 90% confluent they were treated with 2 nM (4 ppm) PFBT nanoparticles for 2 h. Control cells received vehicle alone treatment or treatment with 60 ng/ml interferon- γ and 100 g/ml LPS for 2 h. RNA was extracted and the Total RNA was analyzed by reverse transcriptase PCR for levels of gene expression of the proinflammatory markers TNF α and interleukin-1 β . β -actin was used as a control. We used the following oligonucleotide primers for the RT-PCR: TNF α (forward: 5'-GAACTGGCAGAAGAGGCACT-3'/reverse: 5'-AGGGTCTGGGCCATAGAACT-3'); IL-1 β (forward: 5'-AAATGCCTCGTGCTGTCTGACC-3'/reverse: 5'-CTGCTTGACAGGTGCTGATGTACC-3'), and β -actin (forward: 5'-TGTGATGGTGGAATGGGTCAG-3'/reverse: 5'-TTTGATGTCACGCACGATTTC-3'). Total RNA was extracted (1.0 mL of TRIZOL reagent per 10⁶ cells) according to manufacturer instructions. The isolated RNA was quantified by UV-Vis spectrophotometry (260 nm) and the RNA quality assessed by agarose gel electrophoresis and UV-Vis (260 nm/280 nm ratio). First strand synthesis was carried out with 3 μ g of RNA in a 20 μ L reaction using random hexamers as described by the manufacturer. Amplification of the targeted DNA was carried out in a 50 μ L reaction as instructed by the manufacturer with 4 μ L of the first strand synthesis reaction. The amplified DNA product was separated on a 2% agarose gel with 100 bp DNA ladder as a marker and visualized using SYBR green staining.

RESULTS AND DISCUSSION

In this study we explore the suitability of CP nanoparticles for application in cellular imaging. Using PFBT nanoparticles as representative polymer dots, we examine cellular uptake, chemical toxicity and inflammatory effects in J774A.1 cells, which share many

characteristics of macrophage cells. This cell line allowed investigation of nanoparticle uptake via variety of possible entry routes, since macrophages take up extracellular material by a wide range of mechanisms.

Nanoparticle characterization

PFBT nanoparticles were formed by rapid dilution of conjugated polymer solutions into water, with accompanying sonication. Previous studies have shown that the size of individual particles can be affected by the initial polymer concentration²¹; PFBT nanoparticles used here were prepared by 20-fold dilution of 2000 ppm PFBT (in THF) into water, and then concentrated approximately 4-fold, as described in the Experimental section. Characterization of the resulting PFBT nanoparticles by atomic force microscopy (AFM) indicates that the mean particle height was 18 ± 5 nm (Figure 1). This size is consistent with that previously observed for PFBT particles prepared under similar conditions²¹.

Absorbance and fluorescence emission spectra of the PFBT nanoparticle suspension used in these studies highlight the suitability of these nanoparticles for biological imaging (See Supporting Information; Figure S1). For example, PFBT nanoparticles have a broad absorption spectrum ($\lambda_{\text{max}} = 445$ nm), which provides flexibility in choice of excitation wavelength for imaging applications. The emission maximum for PFBT is centered at 545 nm, a spectral region commonly used for biological imaging. In addition, PFBT nanoparticles have been shown to have little photobleaching at low excitation illumination intensities²¹. The stock nanoparticle concentration used here was estimated to be 140 nM (250 ppm) as described in Experimental. Using this concentration and the measured absorbance at 460 nm, the absorbance cross section (σ) was determined to be 1.8×10^{-13} cm², which is in close agreement with previously reported values²¹.

Nanoparticle dose response and uptake kinetics

To examine CP nanoparticle uptake into cells, we carried out a series of experiments in which J774A.1 cells were incubated in growth medium containing low concentrations of PFBT nanoparticles. Cells were not stimulated by recombinant macrophage colony stimulating factor (rM-CSF) or other pharmacological agents commonly used to induce macropinocytosis or phagocytosis in macrophages; any observed nanoparticle uptake reflects spontaneous processes occurring under approximately physiological conditions. Figure 2A and 2B show differential interference contrast (DIC) and fluorescence images of cells after incubation in media containing 2 nM (4 ppm) PFBT nanoparticles. Intracellular fluorescence appears localized inside the cell, suggesting that PFBT nanoparticles are taken up by the cell.

To determine the efficiency of CP nanoparticle uptake, J774A.1 cells were incubated with varying concentrations of PFBT nanoparticles (loading concentrations of 0.4 – 14 nM/0.7 – 25 ppm) and the resulting fluorescence per cell was determined by flow cytometry. As shown in Figure 2C, each nanoparticle loading concentration tested led to distinct populations of fluorescent cells by flow cytometry, and higher dosing concentrations resulted in greater average fluorescence per cell. At the highest concentrations tested, the observed nanoparticle fluorescence was *ca.* 1000-fold greater than background. Notably, the observed fluorescence per cell was linearly proportional to the dosing concentration (Figure 2D), suggesting that the population of cells was probably capable of taking in higher concentrations of nanoparticles than were tested.

Using the linear relationship between nanoparticle dosing and the observed fluorescence intensity shown in Figure 2D, we calculated the minimum concentration of PFBT nanoparticles required for detection of cells by flow cytometry. Using a standard flow

cytometer with a ≤ 15 mW argon ion laser, PFBT nanoparticle fluorescence in cells could be detected with loading concentrations as low as 155 pM (270 ppb), based on the peak width of the background histogram. This observation highlights the extreme brightness of these nanoparticles, and the resulting very low concentrations required for effective cellular labeling or uptake. Low CP nanoparticle concentrations loaded into cells minimize potential perturbations of the cell biology by the fluorescent marker. Even lower concentrations of PFBT nanoparticles can be detected in cells by fluorescence microscopy, including single nanoparticles²⁶.

To investigate the rate of nanoparticle uptake into cells, cells were incubated with PFBT nanoparticles for different lengths of time, and the resulting intracellular fluorescence per cell was measured by flow cytometry. Figure 3 shows the mean fluorescence intensity as a function of hours of incubation. These data show that nanoparticle uptake is relatively rapid. We were able to see significant uptake within an hour with a PFBT concentration of 14 nM (25 ppm), as shown in Figure 3. Notably, this loading concentration is *ca.* 100 – 1000 times lower than concentrations typically used for fluorescent dextran-conjugate labeling. In addition, as expected, increasing the concentration of extracellular PFBT contributed to higher intracellular fluorescence at a given time point (data not shown).

Figure 3 also demonstrates that CP nanoparticle uptake is strongly temperature dependent. When cells are placed on ice, very little intracellular fluorescence can be observed, even after a long (8h) incubation period. A propidium iodide stained control *fv* showed less than 10% reduction in cell viability at the end of the experiment (Figure S2, see Supplemental Information). Since endocytosis is an active process, uptake by this mechanism slows at low temperature; pronounced reductions in nanoparticle uptake, as observed here, are consistent with internalization via endocytosis, rather than diffusion across the plasma membrane. In addition, the lack of significant nanoparticle fluorescence associated with cells incubated on ice indicates that there is minimal interaction of these nanoparticles with cell surfaces.

Investigation of nanoparticle uptake mechanism

Effective application of nanoparticles as live cell labeling agents requires knowledge of the nanoparticle entry route and final intracellular location. Given the temperature dependence of PFBT nanoparticle loading, we hypothesized that an endocytic mechanism was responsible for the observed PFBT nanoparticle uptake. Indeed, images of intracellular nanoparticle in J774A.1 cells (Figure 2A) suggest that the intracellular fluorescence is contained in vesicles, presumably endosomes, consistent with cell entry through one of the variety of possible endocytic mechanisms. Similar CP nanoparticle staining was also observed in CHO-K1 cells (Figure S3, see Supplemental Information).

As a first step to characterize the intracellular compartments in which CP nanoparticles were localized, we conducted experiments in which cells were incubated in media containing both PFBT nanoparticles and Texas Red dextran (TR-dex; 10,000 MW). Dextran dye conjugates are taken up by host cells via fluid-phase endocytosis, traffic along the endosomal maturation pathway, and finally accumulate in lysosomes³³. As a result, intracellular co-localization of PFBT nanoparticles and TR-dex fluorescence indicates uptake via a shared endocytic mechanism. As shown in Figure 4, fluorescence from PFBT nanoparticles and TR-dex is colocalized. This result suggests that, like TR-dex, CP nanoparticles enter the cell through fluid-phase uptake, and suggests that their final cellular location is the lysosome.

Regardless of the mechanism that controls uptake, extracellular fluid enters the cell in plasma-membrane bounded compartments, early endosomes, which typically mature to late endosomes, before ultimately fusing with lysosomes³⁴. Stages along this pathway are marked by the presence of specific cellular markers in the organelle membrane. For

example, the protein lysosomal associated membrane protein-1 (LAMP-1 or CD107a) is trafficked to the membranes of late endosomes, and remains in the lysosomal membrane. Endosomal trafficking also has a spatial component; early endosomes form near the periphery and move towards the nucleus before they fuse with lysosomes in the perinuclear region of the cell³⁵. To confirm that lysosomes are the final destination of CP nanoparticles, immunofluorescence using an anti-LAMP-1 antibody was performed on PFBT nanoparticle loaded cells to determine whether nanoparticles localized in LAMP-1 containing organelles. Cells were loaded with PFBT nanoparticles, then paraformaldehyde-fixed, permeabilized with detergent, and incubated with an allophycocyanin (APC) labeled anti-LAMP-1 antibody. The resulting images of both PFBT nanoparticles and anti-LAMP-1 antibody fluorescence show localization of CP-nanoparticles in perinuclear organelles that also immunostain with anti-LAMP-1 antibodies (Figure 5). This result indicates that nanoparticles are present in lysosomes and/or late endosomes and confirms that PFBT nanoparticles are taken up by these cells via endocytosis, and ultimately traffic to lysosomes.

A variety of endocytic mechanisms are operative in the cell, including clathrin-mediated endocytosis, caveolin-mediated endocytosis, clathrin- or caveolin-independent endocytosis, and macropinocytosis³⁴. To determine the specific fluid-phase uptake mechanism(s) for CP nanoparticles, we examined the effect of treatment with a series of drugs known to inhibit specific cellular processes required for individual endocytic mechanisms. In these experiments, J774A.1 cells were pretreated with the pharmacological inhibitors at concentrations that resulted in $\leq 10\%$ cytotoxicity (data not shown) and then incubated with PFBT nanoparticles. PFBT entry into individual cells under these conditions was evaluated by measurement of intracellular fluorescence by flow cytometry (Figure 6), and was compared to that for control cells that had been exposed to PFBT nanoparticles in the absence of drug. A decrease in measured nanoparticle fluorescence, compared to the control, reflects involvement of the specific endocytic mechanism targeted by that drug. Moderate but statistically significant inhibition of nanoparticle uptake (*ca.* 30%; $P \leq 0.05$) was observed for nocodazole and cytochalasin D, which inhibit cytoskeletal proteins, including actin. Actin reorganization is necessary for extension of cell membrane to enclose extracellular fluid, and is involved in the majority of characterized endocytic mechanisms. However, no significant reduction in nanoparticle uptake is observed for either chlorpromazine or genistein, which inhibit clathrin-mediated endocytosis. This result indicates that these nanoparticles do not enter the cell via clathrin-coated vesicles. Genistein also inhibits formation of caveolae, thus interfering with entry of CP nanoparticles via caveolae, an endocytic mechanism that results in trafficking to cellular locations other than lysosomes³⁶. Hence, the lack of inhibition observed upon genistein treatment suggests that nanoparticle uptake does not involve caveolin. However, significant inhibition of PFBT nanoparticle uptake (*ca.* 60%) is observed for wortmannin and LY294002, which block the action of phosphoinositide 3-kinase (PI3K). PI3K is required for spontaneous cell surface ruffling that is an integral part of macropinocytosis³⁷. Hence, inhibition of uptake by wortmannin and LY294002 suggests that nanoparticles enter the cell through macropinocytosis. Similarly strong inhibition of nanoparticle uptake is also induced by methyl- β -cyclodextrin, which inhibits cholesterol-dependent endocytosis by extracting cholesterol from the plasma membrane. Cholesterol is involved in membrane ruffling and actin reorganization, among other endocytic functions³⁸. Hence, inhibition of uptake by methyl- β -cyclodextrin is also symptomatic of macropinocytosis, as has been previously reported^{39–41}. Together these data suggest that uptake of CP nanoparticles into these cells occurs via constitutive macropinocytosis rather than clathrin-dependent or caveolin-dependent mechanisms. Immunofluorescence using anti-caveolin-1 and anti-clathrin antibodies were also used to confirm that PFBT nanoparticles were not taken into clathrin or caveolin-1 containing vesicles (See Figures S4 and S5).

Macropinocytosis is a nonspecific mechanism for sampling extracellular fluid⁴² and its contents are delivered to lysosomes within one hour⁴³. During macropinocytosis, cells take in large droplets of the extracellular solution as part of normal physiological function, not as a result of a direct interaction with a cell surface receptor. Entry of PFBT nanoparticles via this mechanism implies that the macrophage cells do not recognize the nanoparticle surface; nanoparticles are simply included into macropinosomes as part of the extracellular soup. In other words, these nanoparticles are inert with respect to the cell surface.

For completeness, phagocytosis should be addressed as a possible mechanism of CP nanoparticle uptake in macrophages. Based on the flow cytometry data in Figures 3 and 6, we have also ruled out phagocytosis as a possible uptake mechanism as it is a receptor-mediated process and the results of the controls with cells incubated on ice strongly preclude a receptor-mediated process. In addition, we have cultured our cells in serum where complement was heat inactivated, thus precluding complement-mediated phagocytosis as the most plausible mechanism of phagocytosis.

Nanoparticle toxicity

Our characterization of PFBT nanoparticle uptake shows that these nanoparticles are efficiently taken into cells via a defined mechanism, an important characteristic for an effective label for live cells and tissues. However, fluorescent nanoparticles should also have low or no observable cytotoxicity, as cells should be unaffected by the imaging probe. To evaluate possible deleterious effects of nanoparticles, the viability of cells loaded with increasing amounts of PFBT nanoparticles was assessed using Cell Titer Blue, which reflects cell viability and proliferation. As shown in Figure 7, the percentage of live J774A.1 cells after incubation with CP nanoparticles for 18 hours was indistinguishable from the control ($P < 0.05$) at all concentrations tested. Nanoparticle dosing in these experiments was limited by achievable nanoparticle concentration in media (≤ 14 nM/25 ppm; see Experimental section), but was more than sufficient for high signal-to-noise imaging applications. Data from these experiments indicate that under these conditions, CP nanoparticles have no discernible impact on cell viability and growth. In addition, images of nanoparticle-loaded cells show a normal morphology and no disruption of actin microfilaments (See Figure S6). Together, these data indicate that CP nanoparticles have no significant cytotoxic effects on this cell line at our maximum working concentrations.

It is possible for nanoparticles to trigger other negative effects unrelated to cell viability and morphology, including changes in the level of gene expression of inflammatory markers^{15, 44, 45}. To explore the possibility of a PFBT nanoparticle induced inflammatory response, we monitored expression of the proinflammatory cytokines tumor necrosis factor α (TNF α) and interleukin-1 β (IL-1 β) in nanoparticle-loaded cells at the mRNA level. As shown in Figure 8, the levels of mRNA coding for TNF α and IL-1 β in the presence of PFBT nanoparticles are identical to those in the absence of nanoparticles. These data suggest that CP nanoparticles do not have an inflammatory effect on this cell type.

Together, these studies of PFBT nanoparticle cytotoxicity indicate that, apart from the presence of nanoparticle fluorescence, cells containing CP nanoparticles were indistinguishable from those without nanoparticles. In other words, in addition to being chemically inert with respect to the cell surface, PFBT nanoparticles appear largely inert with respect to intracellular function. Studies of quantum dot and polymeric nanoparticles have shown that variation of nanoparticle surface coating and charge can affect the pathway of endocytic uptake, as well as the observed toxicity^{14, 46–48}. In other words, for similarly-sized nanoparticles, surface properties determine their respective impact on cellular processes. Since other CP nanoparticles composed of other conjugated polymers share the high hydrophobicity and near-neutral charge of PFBT nanoparticles, we expect that these

other CP nanoparticles under working concentrations would be similarly invisible to normal cell function.

CONCLUSIONS

A variety of endocytic labels are known, including both small molecule organic fluorophores, and nanoparticles of various types. For example, quantum dots, polystyrene and latex spheres, gold nanoparticles, and silica-coated magnetic nanoparticles are also taken up via endocytosis, depending on the surface composition, size, and the cell type^{46, 49–51}. Hence, the mode of entry of CP nanoparticles is comparable to other small molecules and nanomaterials. However, the extremely bright fluorescence of these materials for their size, and the lack of cytotoxicity, is notable. When combined with rapid and efficient cellular uptake, the result is a highly sensitive fluid-phase marker for live cells, with no apparent impact on cellular processes, making CP nanoparticles highly advantageous as fluorescent probes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Characterization of PFBT nanoparticles by AFM. Typical AFM image of PFBT nanoparticles. A) AFM height image; B) histogram of nanoparticle height from AFM image. Mean size = 18 ± 5 nm.

Figure 2. PFBT nanoparticles are taken up by cells in a dose dependent manner

Cells were pulsed with various concentrations of PFBT nanoparticles for 8 hours in DMEM + 10% FBS at 37°C and 5% CO₂ then washed 3X in Ringer's Buffer (RB) prior to imaging or washed 3X in ice cold RB and detached by gentle pipetting prior to flow cytometry. For flow cytometry, 10,000 cells were analyzed for each condition. A) DIC and B) fluorescence micrographs of cells that were pulsed with ~2 nM (4 ppm) PFBT nanoparticles and images acquired at 37°C using a 60X/1.45NA objective, $\lambda_{\text{ex}} = 494$ nm and $\lambda_{\text{em}} = 531$ nm. Scale bar = 10 μm . C) Flow cytometry histograms for cells pulsed with 1 – background, 2 – 850 pM (2 ppm), 3 – 3.5 nM (6 ppm), or 4 – 14 nM (25 ppm) PFBT nanoparticles. Excitation using 488 nm argon ion laser and emission monitored using a 525 nm emission filter. Only 4 of 7 concentrations analyzed are shown for clarity. D) Mean fluorescence intensity from flow

cytometer for all PFBT nanoparticle concentrations tested on cells. The solid line is a linear regression of these data ($R^2 = 0.997$).

Figure 3. Kinetics of PFBT nanoparticle uptake by cells

J774A.1 cells were pulsed with 14 nM (25 ppm) PFBT nanoparticles in DMEM + 10% FBS at 37°C and 5% CO₂ then washed 3X in ice cold RB and detached by gentle pipetting prior to flow cytometry. 10,000 cells were analyzed for each time point. Mean fluorescence intensity from the flow cytometry histograms is shown as a function of time in hours. Error bars represent the standard deviation of the mean fluorescence for at least 3 replicate measurements.

Figure 4. Colocalization of PFBT nanoparticles with TR-dex

Cells were pulsed with 2 nM (4 ppm) PFBT nanoparticles and 250 nM TR-dex overnight in DMEM + 10% FBS at 37°C and 5% CO₂ then washed 3X in RB prior to imaging. Images were all acquired using a images acquired at 37°C using a 60X/1.45NA objective. A) DIC (Scale bar = 10 μm); B) PFBT nanoparticle fluorescence ($\lambda_{\text{ex}} = 494 \text{ nm}/\lambda_{\text{em}} = 531 \text{ nm}$); C) TR-dex fluorescence ($\lambda_{\text{ex}} = 575 \text{ nm}/\lambda_{\text{em}} = 624 \text{ nm}$); D) Merged image; yellow color indicates probable colocalization.

Figure 5. Colocalization of PFBT nanoparticles with LAMP-1

Cells were pulsed with 2 nM (4 ppm) PFBT nanoparticles for 2 hours in DMEM + 10% FBS at 37°C and 5% CO₂ followed by a 4+ hour chase. Cells were then paraformaldehyde fixed, detergent permeabilized, and stained with an anti-LAMP-1 allophycocyanin (APC) conjugated antibody. Images were all acquired at room temperature using a 60X/1.45NA objective. A) DIC (Scale bar = 10 μ m); B) PFBT nanoparticle fluorescence (λ_{ex} = 494 nm/ λ_{em} = 531 nm); C) Cy5 (anti-LAMP-1 stain) fluorescence (λ_{ex} = 575 nm/ λ_{em} = 624 nm); D) Merged image; yellow color indicates probable colocalization.

Figure 6. Blocking cell uptake of PFBT nanoparticles using inhibitors of endocytosis

J774A.1 cells were pretreated for 30 minutes with a series of inhibitors of endocytosis followed by addition of 2 nM (4 ppm) PFBT nanoparticles for 2 hours in DMEM + 10% FBS at 37°C and 5% CO₂ then washed 3X in ice cold PBS and detached by gentle pipetting prior to flow cytometry. Mean fluorescence intensity, which is proportional to CP nanoparticle uptake, is shown for the following treatment conditions: nanoparticles alone, no treatment, nanoparticles on ice, methyl-β-cyclodextrin (2.5 mg/ml), wortmannin (100 ng/ml), LY294002 (20 μg/ml), cytochalasin D (10 μg/ml), nocodazole (10 μg/ml), genistein (10 μg/ml), and chlorpromazine (1 μg/ml). The concentrations of inhibitors used here resulted in ≤ 10% cytotoxicity when run alone as a control using a live-dead assay. Error bars represent the standard deviation of the mean fluorescence for at least 3 replicate measurements. The * indicates a P-value ≤ 0.05 as determined using ANOVA.

Figure 7. Cytotoxicity of PFBT nanoparticles

Cells were grown in black 96-well plates in DMEM + 10% FBS at 37°C and 5% CO₂ and incubated with various concentrations of nanoparticles for 18 hours. The well plate was then read out to record the background from the nanoparticles at $\lambda_{\text{ex}} = 546$ and $\lambda_{\text{em}} = 585$ nm in a top-reading fluorescence plate reader. Cell Titer Blue reagent was then added to all wells and allowed to incubate an additional 2 hours, then the plate fluorescence was recorded again. Individual well background fluorescence was subtracted from the total fluorescence to determine Cell Titer Blue fluorescence and then converted to % viability versus the untreated control wells. The standard deviation is shown based on the average of the wells used for each concentration. These data were also analyzed by ANOVA and all nanoparticle concentrations resulted in P-values > 0.05.

Figure 8. Monitoring inflammatory markers in cells treated with CP nanoparticles

J774A.1 cells were grown in 35 mm culture plates in DMEM + 10% FBS at 37°C and 5% CO₂. When the cells were nearly confluent, they were incubated with 2 nM (4 ppm) PFBT nanoparticles for 4 hours. RNA was extracted from each sample and the total RNA was analyzed for the expression of TNF α , IL-1 β , and actin by RT-PCR and the amplified product separated by agarose electrophoresis. The figure is representative of 3 independent observations. C – vehicle control, Np – 2 nM (4 ppm) PFBT nanoparticles, and In – interferon- γ (60 ng/ml) + LPS (100 ng/ml) as a positive control.