Tailoring cellular uptake of conjugated polymer nanoparticles using modular amphiphilic peptide capping ligands

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KEYWORDS: conjugated polymer nanoparticles, peptide amphiphiles, capping layer, fluorescent nanoparticles, bioimaging, cellular uptake

ABSTRACT: Conjugated polymers possess excellent qualities as fluorescent probes for biomedical applications, because of their extremely high brightness, extinction coefficients, and photostability. Encapsulating these hydrophobic polymers in nanoparticulate form allows transfer to aqueous environments and construction of high-performance fluorescent nanoparticle constructs, and several surface capping strategies have been demonstrated to date. Here, we describe the development of a new class of multifunctional capping ligands for conjugated polymer nanoparticles based on custom-designed amphiphilic peptides. These versatile peptide ligands provide a protective hydrophilic capping layer, chemical handles for further conjugation,

and directed biological activity tuned by altering the specific amino acid sequence. We show that (i) cellular uptake can be regulated as a function of peptide composition, and (ii) the nanoparticles show no signs of toxicity under the conditions used, which is a vital health and environmental issue when developing these technologies for clinical use. Finally, we demonstrate that this one-pot method can be applied to three classes of conjugated polymers and demonstrate potential for multicolor imaging.

Fluorescent probes have proven to be powerful tools for bioimaging and biosensing and have become indispensable in cell biology research. Fluorescence-based detection relies heavily on the properties and performance of the fluorophores used, with brightness and photostability being of particular importance in determining sensitivity and limit of detection. Despite their extensive use, conventional organic fluorophores exhibit broad absorption and emission spectra, have limited brightness, and suffer from rapid photobleaching. This severely constrains their application in high-sensitivity imaging or sensing assays. With the advent of nanotechnology, new fluorescent probes have emerged with vastly improved properties, compared to organic fluorophores. These nanoparticle-based fluorescent probes have received a great deal of interest and have begun to have a significant impact in bioimaging and sensing.

Numerous fluorescent nanoparticles have been developed to date.(1, 2) Silica(3) or polymer(4) nanoparticles can be used to encapsulate large numbers of fluorophores, leading to improved fluorescence brightness and photostability. However, dyes leaching out of such structures and the effects of self-quenching are problematic in these systems. The use of semiconductor quantum dots (QDs) as fluorescent probes for bioassays has drawn the most attention for bioimaging and sensing. The fluorescence emission of QDs can be tuned from the ultraviolet into the infrared by changing their size and/or composition. They exhibit high extinction coefficients and

photostability, and several successful strategies for bioconjugation have been developed. These features, coupled with their broad absorption and narrow emission profiles that allow for optical multiplexing with a single excitation source, make them extremely appealing for imaging and sensing applications.(5, 6) However, issues surrounding their stability in aqueous environments and the cytotoxicity of heavy metal-based QDs(7) remain and these may limit their widespread adoption for bioimaging and sensing.

While initially developed for optoelectronics and photovoltaic applications,(8-11) π -conjugated polymers have recently started crossing into applications in biological systems.(12-15) The π -conjugated structure of conjugated polymers (CPs) yield highly desirable properties—such as very high extinction coefficients and fluorescence brightness—making CPs potentially excellent fluorescent probes in biological applications.

However, in their linear form, CPs offer very limited solubility in water, and although modification with charged side chains can impart solubility, such polymers are poorly protected from undesired interactions in the biological milieu. Encapsulating CPs in nanoparticulate form is an excellent method to overcome these issues, and there has been significant interest in this field of research over the past decade.(14, 15) Conjugated polymer nanoparticles (CPNs) maintain the high fluorescence brightness of CPs; they are photostable, have fast emission rates, and possess low cytotoxicity, making them excellent candidates for advanced biosensing and bioimaging applications.(14, 16) CPNs can be prepared by various methods, most notably the miniemulsion and nanoprecipitation methods, the latter of which having become the standard for CPN formation. In the nanoprecipitation method, CPs are dissolved in a water-miscible “good”

solvent (typically THF) and added rapidly to an aqueous solution under ultrasonic irradiation, and the solvent is subsequently removed. The CPNs form by collapse and coiling of polymer chains due to the sudden change in solvent polarity.(14, 15)

CPNs show great promise in bioimaging and biosensing.(14, 15, 17, 18) In order to maximize these applications, it is imperative that advanced methods of surface capping and functionalization are developed. Current approaches fall into two distinct categories: direct modification of CP backbones, and affinity-driven binding of secondary capping layers. Direct CP modification involves covalent addition of functional groups or biomolecules by modification of the CP monomers, followed by polymerization,(19) or by attachment post-polymerization.(20) An advantage here is the control over the degree of functionalization. However, addition of ionic side groups can adversely affect quantum yield and increased hydrophilicity can impair the formation of tightly packed CPNs as the CPs will tend to keep their linear form in water.(15) Furthermore, direct CP modification requires synthetically challenging and laborious chemical modification of the polymer. In contrast, affinity-driven binding of secondary capping layers relies on interactions, usually hydrophobic, between the CP and a secondary capping agent. An example is the use of phospholipids, originally by Howes et al.,(21, 22) to encapsulate the hydrophobic core in a protective stabilizing layer. Carboxy(polyethylene glycol) functionalized phospholipids were used, where the lipid anchored the molecule in the CPN surface, the PEG offered steric protection of the surface, and the carboxylic acid provided a chemical handle for further modification. An alternative approach uses polymers that possess functional groups in their side chains (for example, polystyrene (PS)-based polymers) that can be coprecipitated with CPs to yield functionalized CPNs. PS-PEG-COOH and poly(styrene-co-maleic anhydride) (PSMA) have both been used by Wu et al.(23, 24) to modify the surface of

CPNs with functional groups to facilitate bioconjugation. In comparison to the direct modification of CPs, it is harder to control the density of functional groups per CPN using a secondary capping method, and it is technically possible for capping agents to leach away over time. Nevertheless, affinity-driven attachment of secondary capping layers is an extremely attractive option as it does not require convoluted synthesis methods of CPs, and allows construction of a large variety of functional fluorescent constructs using standard commercially available CPs.

Herein, we report the preparation of size-controlled functionalized CPNs using custom-designed peptide amphiphiles for one-pot nanoparticle synthesis and functionalization using commercially available CPs. The peptide amphiphiles simultaneously provide a protective hydrophilic capping layer to the particles and chemical handles for further functionalization. Crucially, their modular composition allows direct control over their biological activity by tuning the amino acid sequence. Therefore, we describe herein an improved and multipurpose capping ligand that combines stabilization and functionality in a single molecule. We show that these peptide-CPNs are highly stable with high fluorescence brightness, and demonstrate their versatility in directing biological activity by regulating cellular uptake as a function of amino acid sequence. Finally, we show that our method works for three distinct classes of CP—allowing tuning of optical properties and color of emission—and demonstrate their potential for multicolor imaging.

RESULTS AND DISCUSSION

Peptide-functionalized CPNs were synthesized using a variation of the nanoprecipitation method, as depicted in Figure 1A. CPs are commercially available in a large variety of structures

such that they can be chosen according to the desired optical properties, in particular, the color of fluorescence emission. A polyfluorene CP, poly[(9,9-di-n-octylfluorenyl-2,7-diyl)-alt-(benzo[2,1,3]thiadiazol-4,8-diyl)], referred to hereafter as F8BT, was chosen for use in this work, because of its high fluorescence brightness. Peptide amphiphiles were prepared by conjugation of a branched aliphatic tail to the N-terminus of the amino acid sequence of interest. During the synthesis of peptide-CPNs, the peptide amphiphile is dissolved in water, and then a CP/tetrahydrofuran (THF) solution is added. The rapid change in polarity leads to aggregation of the hydrophobic portions in the core and the orientation of hydrophilic structures into the aqueous phase, yielding CPNs with a hydrophilic peptide capping layer (Figure 1A).

To demonstrate that stable CPNs are formed using amphiphilic peptides with different amino acid sequences, we investigated three different peptides: (1) the TAT sequence (2-hexyldecane-GRKKRRQRRRPQ-amide), a well-known cell penetrating peptide with a positive net charge at physiological pH; (2) an anti-TAT sequence (2-hexyldecane-GDEEDDQDDDPQ-amide), designed to mimic the TAT sequence but with an overall negative net charge; and (3) a zwitterionic PEK peptide (2-hexyldecane-PPPPEKEKEKEK-amide) previously described by Nowinski et al.(25, 26) (Figure 1B). In the original study, the PEK peptide was designed for ultralow fouling, arising because of the formation of a strong hydration layer that resists nonspecific protein adsorption. The structure consists of alternating negatively charged glutamic acid and positive lysine residues, with four proline residues present to provide a compact monolayer on gold nanoparticles, because of hydrophobic interactions.(26) Here, the PEK peptide is used as an inhibitor of cellular uptake (i.e., a “stealth” peptide). For all three peptides, the formation of peptide-CPNs was successful, despite their differing charge characteristics, which proves the versatility of our approach (Figure 2).

The net charge of the peptides during particle synthesis is a key consideration for successful particle formation. It is important that the peptide has a nonzero net charge in the reaction solution; otherwise, the nanoparticles do not form. This is easy to achieve by simply tuning the pH of the water in which the peptides are dissolved prior to particle formation. For the TAT and anti-TAT peptides, particles form at neutral pH. As the net charge of the PEK peptide is zero at neutral pH, the charge equilibrium must be shifted. We observed that nanoparticle formation is not well tolerated under acidic conditions using HCl, possibly due to protonation of the N atoms in F8BT, making it less hydrophobic and therefore impeding the coiling of the CP chains to a certain extent and, hence, nanoparticle formation. Ring-opening and polymerization of THF is also known to occur in the presence of strong acids and might partially account for the failure in the nanoparticle synthesis process under these conditions. Therefore, the PEK/F8BT were synthesized at alkaline pH. The synthesis was performed at pH 12 to ensure full amino acid side group deprotonation and a net negative charge (see Figure S1 in the Supporting Information). Under these conditions, particle formation was successful. The pH of PEK/F8BT was then brought to neutrality prior to use in subsequent experiments.

Peptide-CPNs, made using TAT (TAT/F8BT), anti-TAT (anti-TAT/F8BT), or PEK (PEK/F8BT), were characterized using a combination of techniques. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) (Figures S2 and S3 in the Supporting Information) show that the peptide-CPNs are spherical in form. Figure 2A shows the normalized UV-vis absorption and photoluminescence spectra of the different CPNs in water, showing that the presence of the capping peptides does not perturb their optical properties,⁽²⁷⁾ with peaks at 470 and 535 nm, respectively. Nanoparticle diameters were obtained by dynamic light scattering (DLS), and the nanoparticles harboring the different peptide amphiphiles showed

volume average hydrodynamic diameters of ~ 40 nm (Figure 2B), a size similar to previous reports(28) and suitable for cellular uptake. Figure S4 in the Supporting Information shows the correlation functions and intensity distributions for the DLS measurements. Fourier transform infrared (FTIR) measurements of lyophilized TAT/F8BT, anti-TAT/F8BT, and PEK/F8BT were performed to investigate the presence of the peptides and were compared to the spectrum of the F8BT polymer (Figure 2C). Characteristic peptide bands are seen in all the peptide-CPNs: amide I (C=O, stretch) at $1600\text{--}1690\text{ cm}^{-1}$; amide II (C-N, stretch; N-H, bend) at $1480\text{--}1575\text{ cm}^{-1}$; and at $\sim 3300\text{ cm}^{-1}$ (N-H, bend). Zeta potential measurements (on CPNs in water) provided further evidence of the presence of the respective peptides on the particle surfaces (Figure 2D), with TAT/F8BT showing a positive zeta potential ($+41.1 \pm 12.8\text{ mV}$), and the anti-TAT/F8BT a negative zeta potential ($-42.2 \pm 11.7\text{ mV}$), which is consistent with the charge of each peptide. The zeta potential of the PEK/F8BT was $-19.4 \pm 6.4\text{ mV}$, which matches the value obtained by Nowinski et al.(26) Together, the results in Figure 2 suggest that the peptides are present on the nanoparticle surfaces, and that the optical properties and integrity of the CPNs are not disrupted by their presence. Table 1 shows the quantum yield (QY) values of the TAT/F8BT, anti-TAT/F8BT, PEK/F8BT CPNs, with all CPNs exhibiting similar QY values at ca. 40%.

In addition to the analyses shown in Figure 2, the orientation of the peptides on the CPN surface was investigated by synthesizing PEK with an azide clickable group on a lysine residue at the C-terminus (PEK-N3). PEK-N3/F8BT were produced and after thoroughly washing the excess peptide, propargyl folate was attached to the functional end group of the peptide capping

agent (PEK-FA/F8BT). A folic acid-specific antibody was then used in a dot blot to detect folic acid at the residue closest to the C-terminus of the peptide (Figure 3). This confirms that the hydrophilic amino acid sequence remains on the particle surface, therefore functionalizing the nanoparticle, while the hydrophobic portion of the peptide structure interacts with the hydrophobic CP and becomes embedded in the CPN surface, anchoring the peptide in place. Furthermore, it confirms the bioavailability of the capping peptide.

To investigate the colloidal stability of the peptide-CPNs, DLS measurements of the particles in water were carried out over a period of 15 days (Figure S5 in the Supporting Information). All peptide-CPNs demonstrated high stability in water without significant changes in DLS volume distribution or volume average diameter over a period of 15 days, with diameter centered around ca. 50 nm. To give an indication of the behavior of the peptide-CPNs in the culture media (Dulbecco's Modified Eagle medium (DMEM) supplemented with 1% (v/v) fetal bovine serum), we incubated them in media and analyzed the solution using DLS. Figure S6 in the Supporting Information shows the DLS intensity distributions at 0, 2, and 48 h, which show their primary peaks at ca. 100 nm and above. These results suggest there is a degree of protein adsorption on the particle surfaces, but that the particles maintain colloidal stability. The nature of the particles (i.e., protein composition and strength of protein binding) has not been studied. Thus, it is important to note that, as is the case with all nanoparticle–cell interactions, what the cell perceives is a product of the surface chemistry of the nanoparticle, and the material associated with it.

To demonstrate that the biological activity of the peptide-CPNs can be specifically directed by altering the composition of the peptide capping layer, we studied their interaction with cells. The

effect of the peptide composition on cellular uptake of peptide-CPNs was evaluated in HeLa cells by confocal laser scanning microscopy and flow cytometry. Figure 4 shows the differences in cell uptake after incubation with peptide-CPNs for up to 24 h in DMEM supplemented with 1% (v/v) FBS. The concentration of all CPNs was adjusted in the stock solutions by recording and matching their absorbance at 470 nm and a final concentration of 0.5 nM was calculated. The TAT/F8BT, anti-TAT/F8BT, and PEK/F8BT stock solutions were then diluted 10-fold (final concentration of 0.05 nM) in DMEM supplemented with 1% (v/v) FBS for incubation with HeLa cells.

Confocal microscopy images were recorded at times of 30 min, 2 h, 6 h, and 24 h post-CPN incubation, as representative time-points (Figure 4A). For TAT/F8BT, the CPNs start to be internalized within 30 min of incubation and can be observed in the perimembraneous region of the cells. After 2 h, the TAT/F8BT start to migrate inside the cells from the outer cell membrane to the cytoplasmic region, and are seen mainly inside the cells at 6 h post-incubation. The strong green fluorescence that is observed indicates that TAT/F8BT is easily internalized. TAT/F8BT accumulates in the perinuclear region of the cells after 24 h of incubation. In contrast, no uptake of anti-TAT/F8BT and PEK/F8BT is observed by confocal microscopy until after 24 h of incubation with cells. At this time-point, it is noticeable that some CPNs are internalized and, in this case, they also are in the perinuclear region of the cells.

Flow cytometry can be performed without fluorescent labeling, because of the high fluorescence brightness of F8BT, and we used it as a semiquantitative tool to facilitate relative comparisons of cellular uptake at various time-points and with different peptide-CPNs. Time-points for 30 min, 2 h, 6 h, 9 h, and 24 h post-CPN incubation were chosen for flow cytometry analysis. The results suggest that the uptake of TAT/F8BT is preferential, with a 10-fold higher

uptake versus anti-TAT/F8BT and PEK/F8BT after only 30 min of incubation, with this preferential uptake continuing throughout the incubation time (Figure 4B). Uptake of anti-TAT/F8BT and PEK/F8BT is minimal, in comparison to TAT/F8BT, which is expected as previous reports show that highly positively charged peptides facilitate internalization(29, 30) while negatively charged peptides and negatively charged amino acids between positively charged amino acids inhibit cellular uptake.(29) The flow cytometry data in Figure 4B also shows that anti-TAT/F8BT CPNs are internalized more than PEK/F8BT CPNs. The low internalization of the PEK/F8BT is in accordance with the stealth-like properties of the PEK peptide. In order to contextualize the performance of the PEK peptide, we synthesized PEG-NH2/F8BT CPNs using a PEGylated phospholipid and the same synthesis conditions as those used for the peptide-CPNs. This provided an important control as PEG-capped nanoparticles are the current standard for avoiding protein adsorption and uptake by cells.(31) In our study, the HeLa cells showed only residual internalization of the PEG-NH2/F8BT, even after 24 h of incubation (Figure 4B).

Although PEG-NH2/F8BT showed lower fluorescence intensity values than PEK/F8BT, the level of internalization of these CPNs is comparable. We hypothesize that the small differences might be explained by the ca. 10% lower QY of the PEG-NH2/F8BT CPNs, when compared to all the peptide-CPNs, thereby lowering the fluorescence intensity measured by flow cytometry, compared to an equivalent number of peptide-CPNs.

Previous studies on CPN uptake by cells, including uncapped,(32) PEGylated,(22) and CP-loaded PLGA nanoparticles,(33) typically point to common endocytotic pathways. To further understand how the TAT/F8BT are internalized by HeLa cells, a mechanistic study was performed using inhibitors of the main endocytic pathways. Six inhibitors were used:

wortmannin (blocks the activity of phosphoinositide 3-kinase, a key regulator in macropinocytosis), cytochalasin D (actin polymerization inhibitor), nocodazole (microtubule polymerization inhibitor), chlorpromazine (blocking agent of clathrin-coated pit formation), filipin III (inhibitor of caveolae formation), and methyl- β -cyclodextrin (cholesterol-lowering agent) (see Figure 5A). The HeLa cells were treated with each inhibitor individually and a significant decrease in TAT/F8BT uptake was observed for cytochalasin D, nocodazole, and chlorpromazine (see Figure 5B). These inhibitors interfere with the micropinocytic pathway and the decrease with chlorpromazine indicates a clathrin-dependent uptake. These results are consistent with previous studies with the TAT peptide.(34)

In order to demonstrate the biocompatibility of the peptide-CPNs with HeLa cells, cytotoxicity assays were performed. The Alamar Blue assay was performed to measure cell proliferation and the lactate dehydrogenase (LDH) assay to measure the release of the LDH enzyme as an indicator of cellular death (see Figure 6). After 24 h of incubation with the HeLa cells under the conditions and concentrations chosen, the peptide-CPNs used in this study did not show cytotoxicity to cells, since their metabolic activity and LDH production showed no statistical significant differences, relative to that of the HeLa cells in absence of CPNs. The production of the LDH enzyme is slightly elevated for the PEK/F8BT, but this difference is not statistically significant. The lack of cytotoxicity presented in Figure 6 demonstrates that the differences observed in cellular uptake are due to differences in the nature of the peptides and not cytotoxic effects on the cells that could hinder nanoparticle uptake.

Simultaneous detection of multiple probes in a single sample is highly desirable in both bioimaging and biosensing. To demonstrate the versatility of our synthesis process, the formation of peptide-CPNs was tested using CPs with different emission wavelengths and chemical

compositions. CPs belonging to three distinct classes—polyfluorene (F8BT), polyvinylene (poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene], MEH-PPV) and polyethylene (poly[2,5-di(3',7'-dimethyloctyl)phenylene-1,4-ethynylene], PPE)—were capped by TAT, forming TAT/F8BT, TAT/MEH-PPV, and TAT/PPE, respectively. In each case, nanoparticle formation was successful, yielding particles of a similar size (ca. 40 nm). Figure 7 shows the optical characteristics and DLS hydrodynamic diameter of the TAT-CPNs (corresponding DLS correlation functions and intensity distributions are shown in Figure S7 in the Supporting Information). FTIR spectroscopic analysis of all TAT-CPNs exhibited characteristic peptide peaks, indicating the presence of the peptide on the CPNs (see Figure S8 in the Supporting Information). TAT/PPE were added to HeLa cells under the same conditions as those previously used with TAT/F8BT (Figure S9 in the Supporting Information), and a similar cellular trafficking was observed, despite the change in CP.

We demonstrate the potential for multicolor imaging by simultaneous incubation of TAT/PPE and anti-TAT/F8BT with HeLa cells. The CPNs internalization was followed by confocal microscopy at 2, 6, and 24 h and the same pattern of internalization can be observed for the TAT-capped CPNs: TAT/PPE can be seen in the perimembranous region of the cells after 30 min of incubation with cells and they move to the cytoplasmic region (2 and 6 h after incubation) and finally can be seen in the perinuclear region after 24 h of post-incubation (see Figure 8). However, when anti-TAT/F8BT are incubated together with the TAT/PPE, these CPNs get internalized quickly, as opposed to what was observed when they were incubated with HeLa cells on their own, under the same conditions (Figure 4A). After 30 min of incubation of HeLa cells with both CPNs, anti-TAT/F8BT can be observed at the perimembranous regions of the

cells, along with the TAT/PPE, although it appears that anti-TAT/F8BT is present at this stage in smaller numbers than the TAT/PPE. The movement to the cytoplasmic area occurs at 2 h and is more pronounced at 6 h for both CPNs, and colocalization can be observed most likely due to entrapment in lysosomes. As observed previously, at 24 h, both CPNs are located around the nuclei of the cells (Figure 8). This piggyback effect of anti-TAT/F8BT on TAT/PPE can probably be explained by their opposite capping ligand charges: anti-TAT, being negatively charged, can form electrostatic bonds with TAT and, because TAT is a cell penetrating peptide, TAT/PPE facilitates anti-TAT/F8BT internalization. The employment of cell-penetrating peptides has been used extensively to translocate covalently or electrostatically bound cargo, such as nucleic acids,(35, 36) proteins,(37) or imaging agents,(38) that otherwise would not be easily internalized by cells (due to the negative charges of both the cargo and the cell membranes or the high molecular weight of the cargo). Therefore, it is possible that TAT-capped nanoparticles are also able to translocate other nanoparticles coated with a negatively charged ligands without covalent attachment.

CONCLUSIONS

CPNs have emerged as very promising fluorescent probes for biosensing and bioimaging, as alternatives to organic dyes and other fluorescent nanoparticles. Here, we present a one-pot synthesis method of functionalized CPNs based on hydrophobic interactions between the CP core and a branched aliphatic tail at the N-terminus of a peptide. Peptides provide several advantages over other commonly used capping ligands, since they are biocompatible and can be bioactive, are nonimmunogenic, have polarity that can be easily adjusted, are easily conjugated to other molecules, and are biodegradable. This new route provides a universal way of anchoring peptides to CPNs and combines CPN stabilization with bioactivity. Notably, we demonstrate

that, by tuning the amino acid sequence of the peptide amphiphile, we can trigger a different cellular uptake response, accelerating or decelerating internalization of the peptide-CPNs. We also show that the color of the emission can easily be changed by altering the constituent CP, and that peptide-CPNs harboring different peptides and CPs can be used for multicolor imaging. Furthermore, when one of the CPNs is coated with the TAT cell-penetrating peptide and the other CPNs are capped with a negatively charged peptide, the TAT-capped CPNs facilitate the internalization of the latter. We also demonstrate that our system does not present cytotoxicity to the HeLa cells under the conditions used, fulfilling an important requirement for the implementation of these nanoparticles in biomedicine. We envision that, because of the decreased distance between the functional moieties and the CP, this method also holds great potential for application in biosensing.

MATERIALS AND METHODS

Materials

All of the chemicals and solvents were purchased from Sigma–Aldrich, unless otherwise stated.

The nanoparticle size and zeta-potential measurements of the CPNs in solution were performed by DLS, using a ZetaSizer Nano ZS (Malvern). UV-vis absorption and fluorescence spectra were acquired using an Envision plate reader (Perkin–Elmer). IR spectra were recorded with a Spectrum 100 Fourier transform infrared spectrophotometer (Perkin–Elmer).

Synthesis of Peptide Amphiphiles

The peptides were synthesized on a Symphony Quartet (Ranin) automated peptide synthesizer using a standard Fmoc-solid phase protocol. The branched alkyl chain was added manually to each peptide on resin using 2-hexyldecanoic acid. Subsequently, peptides were cleaved off resin with a solution of 95% (v/v) TFA, 2.5% (v/v) TIS, 2.5% (v/v) water for 4 h. TFA was then removed under reduced pressure and the peptides precipitated in cold diethyl ether and dried under vacuum. The amphiphilic peptides were purified by reverse-phase high performance liquid chromatography (HPLC) (Shimadzu) with a linear gradient of H₂O/ACN solution containing 0.1% (v/v) TFA (2-hexyldecane-TAT and 2-hexyldecane-PEK) or 0.1% (v/v) NH₄OH (anti-TAT). A Phenomenex C18 Gemini NX column with 150 mm × 21.2 mm, a 5 µm pore size, and 100 Å particle size was used. The molecular weight of the peptide amphiphiles was determined by mass spectrometry. The peptide amphiphiles were stored at -20 °C until further use.

CPN Synthesis and Functionalization

F8BT polymer was used to produce the CPNs by the reprecipitation method: 2 mL of a solution of 10 µg/mL of F8BT in THF were injected in 10 mL of water under ultrasonication. The amphiphilic peptide capping agent (TAT, anti-TAT, PEK) or PEG-NH₂ (Avanti Polar Lipids) was added to the water prior THF addition at a concentration of ~25 µM. The THF was removed under reduced pressure and the excess capping agent was removed by repeated centrifugation cycles at 4000 g using a 10 kDa Amicon spin-filter tube. The same synthesis procedure was followed when PPE and MEH-PPV were used.

Calculation of CPN Concentration

To calculate the NPs concentration in the stock solutions, the total number (N) of CPNs was first estimated, where the total amount of polymer (m) divided by the density of CPNs (ρ) gives the total CPNs volume, and the average CPN volume can be determined by the mathematical formula for the volume of a sphere, with the average radius (r) being determined by DLS.

$$N = \frac{\text{total CPNs volume}}{\text{average CPN volume}} = \frac{\frac{m}{\rho}}{\frac{4}{3}\pi r^3}$$

Thus, considering an initial mass of polymer used of 20 μg , a density of $\sim 1 \text{ g/cm}^3$, and radius of 20 nm, the value of N is 6×10^{11} . This, in turn, can be used to calculate the final concentration of a CPN batch, using the expression

$$[CPN] = \frac{N}{\frac{6.02 \times 10^{23} \text{ mol}^{-1}}{v_f}}$$

The parameter v_f corresponds to the final volume of the CPN stock, which was 2 mL for all batches; therefore, a concentration of $\sim 0.5 \text{ nM}$ was used for all nanoparticles.

Quantum Yield Measurements

Quantum yield (QY) values were measured by comparison with fluorescein (in 0.1 M NaOH, QY = 0.79) as a fluorescence standard. Five dilutions were prepared for each CPN batch (TAT/F8BT, anti-TAT/F8BT, PEK/F8BT, and PEG-NH₂/F8BT) and the standard, where the most concentrated sample for each batch had a maximum absorption of 0.1 at 470 nm. UV-vis absorbance spectra and emission spectra (excitation wavelength of 470 nm) were recorded for all samples and standards. The absorption at 470 nm versus the integral of emission spectra for each

dilution and sample and the standard were plotted and trend lines fitted. The slope (m) values were used to calculate the QY of each sample, according to

$$QY_{sample} = QY_{standard} \times \frac{m_{sample}}{m_{standard}} \times \frac{\eta_{sample}^2}{\eta_{standard}^2}$$

Dot-Blot Immunoassay

PEK/F8BT and PEK-FA/F8BT at the same concentration were deposited in a nitrocellulose membrane. To minimize the area that the solution penetrates, spots of 5 μ L were added slowly and allowed to dry before any subsequent increases in concentration were performed by addition of 5 μ L more in the same area. Five concentrations were used for each, where the concentration of a given spot was 5 μ L higher than the previous spot. When fully dry, nonspecific sites in the membrane were blocked by soaking in 5% (w/v) bovine serum albumin (BSA) in 20 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween20 at pH 7.6 (TBST) for 1 h at room temperature. Then, monoclonal antifolic acid primary antibody diluted 1:500 in 5% (w/v) BSA in TBST was added to the membrane for 1 h at room temperature. The membrane was thoroughly washed with TBST (three times, for 5 min each) and IgG IRDye secondary antibody (LI-COR Biosciences) in TBST was added to the membrane for 1 h at room temperature and protected from the light. Afterward, the membrane was thoroughly washed with TBST (three times, for 5 min each) and imaged using an Odyssey infrared scanner and ImageStudio software (LI-COR Biosciences).

CPN Uptake Time-Course

HeLa cells were seeded using DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics/antimycotics (A/A) mix in a six-well plate and an eight-well μ -Slide (Thistle Scientific) per time-point at a density of 7×10^4 cells/mL 24 h prior to the experiment. CPN batches harboring the different peptides were also prepared and characterized 24 h before the experiment with concentration measured by the absorbance at 470 nm and, if needed, corrected to ensure that all batches were at the same concentration (0.5 nM). On the day of incubation with the nanoparticles, CPNs were added to DMEM supplemented with 1% (v/v) FBS and 1% (v/v) A/A mix to a final concentration of 0.05 nM and incubated with the HeLa cells. Control samples with the same volume of water added instead of nanoparticles permitted the exclusion of any possible media dilution interference on the results. Results for six time-points were collected: 30 min, 2 h, 4 h, 6 h, 9 h, and 24 h after incubation with the different nanoparticles. At each time-point, the cells seeded in the eight-well μ -Slide were fixed with 4% (v/v) paraformaldehyde (PFA) in phosphate buffered saline (PBS), permeabilized in 0.1% (v/v) Triton X-100 for 5 min at room temperature, rinsed with PBS twice and stained for the cytoskeleton (actin filaments) with AlexaFluor 647 phalloidin (Life Technologies), and nuclear staining with DAPI. Confocal imaging was performed on a Leica SP5 resonant inverted confocal microscope (Leica GmbH). The cells seeded in the six-well plates were trypsinized using 0.05% (w/v) trypsin-EDTA, followed by the addition of media, centrifuged at 500 g for 5 min. The supernatant was removed and the pellet resuspended in 4% (v/v) PFA in PBS and filtered through a 40 μ m mesh to remove possible cell aggregates. Samples were analyzed in a BD LSRFortessa cell analyzer (BD Biosciences).

Uptake Mechanism Studies

HeLa cells were seeded using DMEM supplemented with 10% (v/v) FBS and 1% (v/v) A/A mix in six-well plates at a density of 7×10^4 cells/mL and a batch of 2-hexyldecane TAT-functionalized CPNs 24 h prior to the experiment. The cells were incubated for 1 h with DMEM supplemented with 1% (v/v) FBS and 1% (v/v) A/A mix and cytochalasin D (10 μ g/mL), nocodazole (10 μ g/mL), methyl- β -cyclodextrin (5 mg/mL), chlorpromazine (10 μ g/mL), filipin III (0.5 μ M), and wortmannin (0.1 μ g/mL). The media was then substituted with fresh media containing the inhibitors at the same concentrations and 0.05 nM TAT/F8BT CPNs and incubated for 1 h. Cells without any inhibitor and cells without inhibitors but with CPNs served as negative and positive controls for the experiment, respectively. Subsequently, the cells were processed for flow cytometry analysis (vide supra). To ensure that the effects observed on the uptake were unrelated to toxic effect on the cells, each inhibitor at the concentrations used for the assay was tested. Alamar Blue and LDH assays were performed as done previously (vide infra).

CPN Toxicity Studies

To measure HeLa cell viability in the presence of peptide-CPNs, culture medium was replaced with DMEM containing 10% (v/v) Alamar Blue and the HeLa cells were incubated for 1 h at 37 °C in the presence of 5% CO₂. The plate was then read on a microplate reader at 570 and 600 nm (reference wavelength). To perform the colorimetric lactate dehydrogenase (LDH) assay, culture media from each well were collected and incubated with an equal volume of substrate mix in assay buffer from the CytoTox-ONE kit (Promega) for 1 h at room temperature and

protected from direct light. A stop solution from the same kit was added after this time to stop the reaction. A solution of DMEM with 0.1% (v/v) Triton X-100 was used as 100% lysis control and was added to the relevant wells 45 min prior to the supernatant harvest. All cytotoxicity studies were performed after 24 h of incubation with peptide-CPNs and under the same conditions as for the cellular uptake studies.

FIGURES

Figure 1. (A) Schematic representation of the nanoprecipitation method for the synthesis of peptide-CPNs with TAT, anti-TAT, and PEK peptides, and F8BT polymer. (B) Chemical structures of the TAT, anti-TAT, and PEK peptides.

Figure 2. Characterization of the peptide-CPNs. (A) Normalized absorption (solid lines) and emission (dashed lines) spectra of peptide-CPNs in water; (B) DLS volume distribution curves of peptide-CPNs in water; (C) FTIR spectra of lyophilized peptide-CPNs and F8BT polymer; and (D) ζ -potential data for peptide-CPNs in water. Legend: red corresponds to data for TAT/F8BT, blue corresponds to data for anti-TAT/F8BT, and green corresponds to data for PEK/F8BT; black, when present, shows the data for the F8BT polymer.

Figure 3. Dot-blot immunoassay for fluorescent detection of folic acid. Peptide-CPNs were deposited at the same concentration, and the concentration of each sample is double that of the previous sample. The graph represents the intensity of each spot: white circles correspond to PEK/F8BT, and black circles correspond to PEK-FA/F8BT.

Figure 4. Cellular uptake of peptide-CPNs by HeLa cells in DMEM supplemented with 1% (v/v) FBS. (A) Representative confocal images of TAT/F8BT, anti-TAT/F8BT, and PEK/F8BT incubation with HeLa cells at 30 min, 2 h, 6 h, and 24 h (scale bars correspond to 50 μ m; actin

staining is shown in red, nuclei staining is shown in blue, and peptide-CPNs fluorescing is shown in green). (B) Normalized flow cytometry data intracellular uptake in HeLa cells at 30 min, 2 h, 6 h, 9 h, and 24 h for all peptide-CPNs; red represents TAT/F8BT, blue represents anti-TAT/F8BT, green represents PEK/F8BT, and gray represents PEG-NH2/F8BT.

Figure 5. (A) Schematic representation of major pinocytic pathways of nanoparticles in mammalian cells and of the inhibitors used for each pathway. (B) Flow cytometry data of intracellular uptake in HeLa cells after 2 h of TAT/F8BT incubation in DMEM supplemented with 1% (v/v) FBS. [Legend: (*) $P < 0.05$, (**) $P < 0.001$, and (***) $P < 0.0001$.]

Figure 6. Determination of cytotoxicity of peptide-CPNs to HeLa cells with Alamar Blue and LDH assays after 24 h: (A) data for Alamar Blue, expressed relative to untreated controls, and (B) data for LDH are relative to the maximum LDH leakage (100% lysis). [Legend: (***) $P < 0.0001$.]

Figure 8. Cellular uptake of TAT/PPE and anti-TAT/F8BT by HeLa cells in DMEM supplemented with 1% (v/v) FBS. Confocal microscopy images at 30 min, 2, 6, and 24 h of incubation with CPNs. [Scale bars represent 50 μ m. Actin staining is represented in red, TAT/PPE fluorescing is represented in blue, and anti-TAT/F8BT fluorescing is represented in green.]

TABLES

Table 1. Quantum yield data for the peptide-CPNs.

Peptide-CPN	QY
TAT/F8BT	37%
Anti-TAT/F8BT	42%
PEK/F8BT	37%

ASSOCIATED CONTENT

Supporting Information. Net charge curve at different pH values for the PEK peptide, stability of peptide-CPNs in water and media measured by DLS over time, FTIR spectra of TAT/PPE, TAT/MEH-PPV, PPE polymer and MEH-PPV polymer, fluorescence image of HeLa cells incubated for 30 minutes with TAT/PPE, and NMR of propargyl folate. Also, additional methods are provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

The authors would like to acknowledge Stephanie Maynard for help with confocal microscopy. C.S.A. was supported by the FCT doctoral fellowship SFRH/BD/80544/2011. I.K.H. acknowledges the Swiss National Science Foundation (SNF grant no. 145756). P.D.H. acknowledges support from the Engineering and Physical Research Council (EPSRC, UK). M.M.S. acknowledges support from EPSRC through the Interdisciplinary Research Centre (IRC) “Early-Warning Sensing Systems for Infectious Diseases” (EP/K031953/1) and research grant “Bio-functionalised Nanomaterials for Ultrasensitive Biosensing” (EP/K020641/1). Optical microscopy was performed in the Facility for Imaging by Light Microscopy (FILM) at Imperial College London.

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