

# Nanoparticles for Drug Delivery Prepared from Amphiphilic PLGA Zwitterionic Block Copolymers with Sharp Contrast in Polarity between Two Blocks\*\*

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The stability and targeting efficiency of nanoparticles (NPs) are the two most important factors for their successful application to drug delivery and diagnostic imaging.<sup>[1]</sup> Coating of the materials is needed to render NPs both stable and multifunctional to accommodate these two factors. Owing to its resistance to nonspecific protein adsorption (or nonfouling properties), polyethylene glycol (PEG)<sup>[2–12]</sup> is the material most commonly used to modify NPs to make them stable.<sup>[2,3,5,6]</sup> However, PEG is susceptible to oxidative damage and therefore limits its long-term applications.<sup>[13–18]</sup> Besides their stability in complex media, the stability of the NPs themselves is another important factors which is often overlooked. NPs need to remain intact throughout any necessary manufacturing processes such as centrifugation or lyophilization. To maintain the stability of NPs, including those coated with PEG, several measures must be used such as low-speed ultrafiltration and addition of cryoprotectants prior to freeze-drying.<sup>[19–21]</sup> For targeted drug delivery, biorecognition elements (e.g. targeting ligands) often need to be immobilized onto NP surfaces. There is only one functional group potentially available at the end of a long PEG chain (e.g. 2–5 kDa) to which biomolecules can be conjugated.<sup>[11,12]</sup> In addition, functional groups that have not reacted can cause nonspecific binding, particularly in complex media such as blood plasma and serum.<sup>[22]</sup> With all current materials used for NP coating, there is a compromise between excellent stability and multifunctionality. To the best of our knowledge, a single material or coating that can accommodate both NP ultrastability and multifunctionality does not exist.

Herein, we present a specific type of zwitterionic material, poly(carboxybetaine) (PCB; Figure 1), which is unique in that each CB side chain has one carboxylate anion that can be used for conjugation with amine-containing biomolecules.<sup>[23–26]</sup> At the same time, each carboxylate anion is paired with one cationic quaternary amine as a zwitterionic unit, and this pairing effectively resists nonspecific protein

adsorption even from complex media.<sup>[25–27]</sup> The conjugation with biomolecules can be easily achieved through 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and *N*-hydroxysuccinimide (EDC/NHS) chemistry. After conjugation, remaining ester groups of NHS are hydrolyzed to carboxylate anions, which are paired with cationic quaternary amines to form nonfouling zwitterionic structures. However, for functionalizable PEG terminated by COOH groups, remaining functional groups (e.g. carboxylic acid) can cause severe fouling problems, particularly in complex media. Thus, PCB has abundant functional groups and an ultra-low fouling background all in one material. This material does not compromise between the two quite different properties, that is, nonfouling and functionalization. When antibodies are immobilized on a PCB-coated sensor chip, the postfunctionalized surface still maintains the ultra-low fouling properties ( $<0.3 \text{ ng cm}^{-2}$  adsorbed protein even from undiluted blood plasma and serum) and biomarkers in blood can be detected with high sensitivity.<sup>[25]</sup> It is expected that introduction of CB onto NP surfaces will improve both the stability and the multifunctional abilities of drug-delivery carriers. This idea is tested in a poly(lactic-co-glycolic acid) (PLGA)-based drug delivery system. Already approved by the FDA, PLGA has been used to encapsulate and control drug release because of its hydrophobic and slow hydrolysis nature in aqueous media.<sup>[28]</sup> We have designed and developed PLGA-PCB block copolymers that self-assemble into NPs with a PLGA core and a PCB shell. These NPs could be used for targeted drug delivery.

In examining PLGA-PCB NPs, we were interested in any stabilizing effects from the strong hydration of zwitterionic CB and the sharp hydrophilicity/hydrophobicity difference between the PLGA core and the PCB shell. This difference between the two blocks is so great that it is very difficult to find a solvent or mixed solvent system to co-dissolve both PLGA and PCB homopolymers. Thus, the synthesis of PLGA-PCB block copolymers is very challenging as a result of these solubility problems. To solve this problem, we designed a new carboxybetaine monomer containing a *tert*-butyl ester group (CB-*t*Bu; Figure 1). Unlike zwitterionic CB, the CB-*t*Bu monomer is a cationic ester with good solubility in organic solvents, thus enabling the covalent binding of PLGA with PCB-*t*Bu polymers in a common solvent, such as acetonitrile. Zwitterionic CB molecules can be regenerated by hydrolysis of the *t*Bu ester groups in an acid environment (TFA) after covalent bonding with PLGA. For the first time, this synthetic route broadens the applicability of zwitterionic CB molecules in organic synthesis, thereby making reactions

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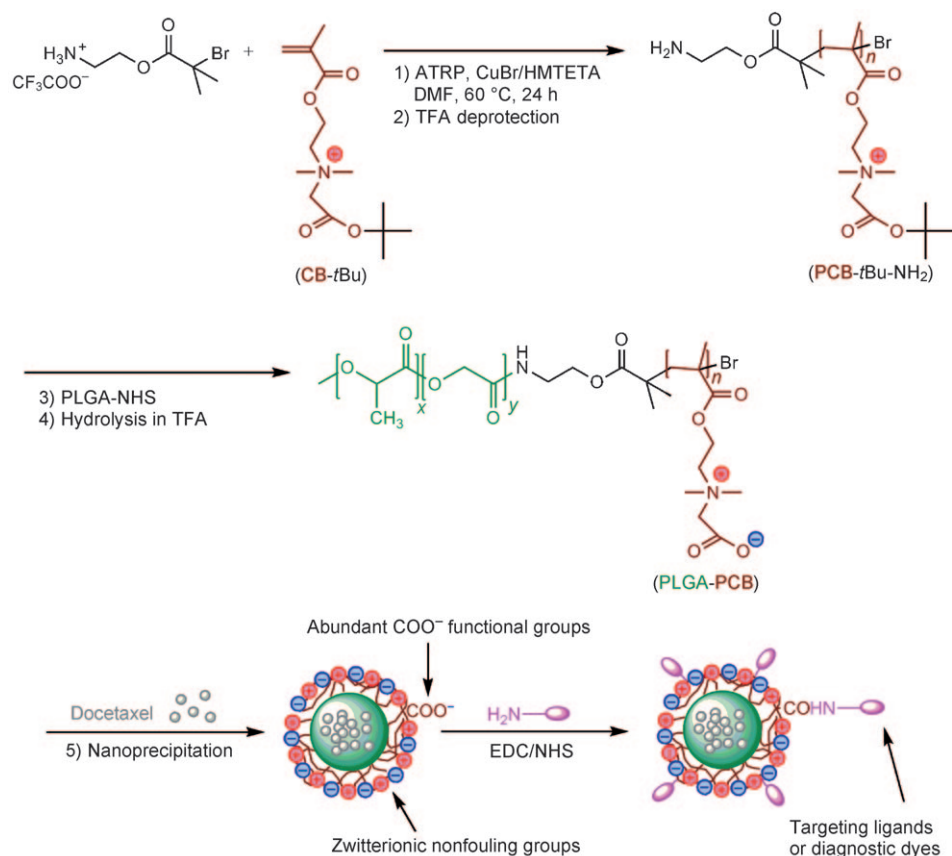
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between polar, zwitterionic CB and a wide range of hydrophobic molecules possible.

Herein we describe the synthesis of PLGA-PCB block copolymers, which have the greatest polarity contrast between blocks ever studied. It is further demonstrated that NPs capable of drug delivery and assembled from such copolymers—with sharp polarity differences—exhibit unusual properties. These properties are rarely found in current NP systems, but are essential to practical applications; these NP systems have high stability in biological media, are easy to manufacture, and are amenable to functionalization.

The PLGA-PCB block copolymers may be prepared either through radical polymerization of CB initiated by PLGA (with appropriate terminal group) or by the conjugation of PLGA and PCB (i.e. PLGA terminated by COOH groups coupling with PCB terminated by NH<sub>2</sub> groups). The latter route is preferred because the NH<sub>2</sub>-PCB products can be conjugated to a wide range of molecules terminated by COOH groups, including the commercially available PLGA used in this work, potential chemotherapy drugs, and proteins or enzymes for stabilization and multifunctionalization purposes. However, direct conjugation of NH<sub>2</sub>-PCB to COOH-PLGA blocks is difficult because of their dramatic difference in polarity. Zwitterionic PCB can be only soluble in water or methanol, whereas PLGA is not soluble in either solvent. Also, it is known that PLGA can be hydrolyzed by trace amounts of water. To solve these “solvent” problems, we designed a CB-*t*Bu ester monomer, which is stable and has good solubility in anhydrous organic solvents such as acetonitrile and DMF. These CB-*t*Bu ester monomers are polymerized by an atom transfer radical polymerization (ATRP) method initiated by an initiator bearing TFA-NH<sub>3</sub><sup>+</sup> (2-aminoethyl 2-bromoisobutyrate; Figure 1, Step 1). After removal of the TFA salt, PCB-*t*Bu-NH<sub>2</sub>, which has good solubility in organic solvents, was obtained (Figure 1, Step 2), thus enabling conjugation with PLGA-NHS in anhydrous acetonitrile to form PLGA-PCB-*t*Bu block copolymers (Figure 1, Step 3). The uniqueness of the *t*Bu ester group lies in the easy removal of this group by TFA to generate a zwitterionic CB structure while the PLGA remains intact, even in an acidic environment such as the one employed (Figure 1, Step 4). We found that treatment with TFA for 1 hour was sufficient to fully convert PCB-*t*Bu into



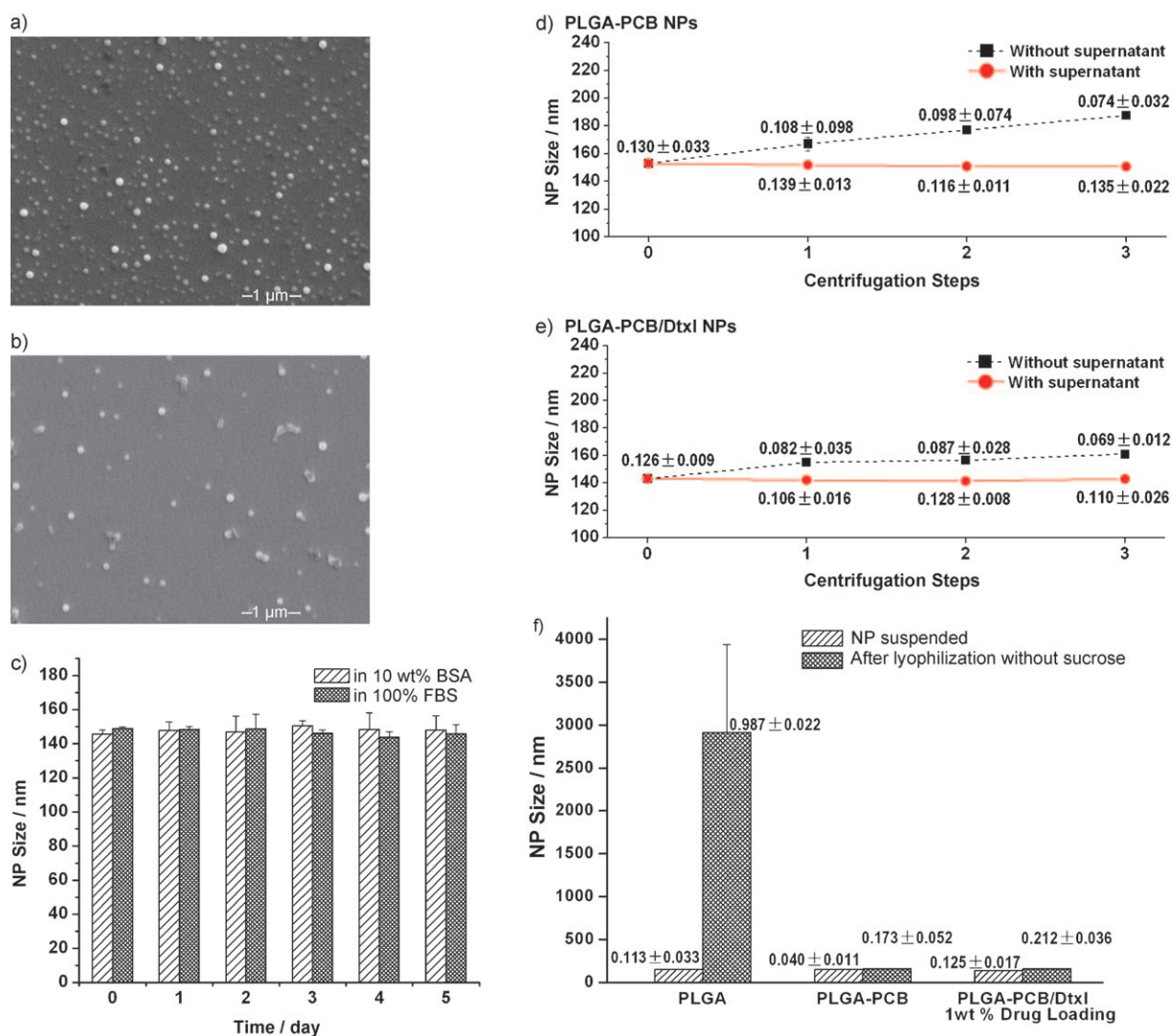
**Figure 1.** Synthesis of PLGA-PCB copolymers, formation of PLGA-PCB/Dtxl NPs, and postfunctionalization of NPs with targeting ligands or diagnostic dyes. DMF = *N,N*-dimethylformamide, HMTETA = 1,1,4,7,10,10-hexamethyltriethylenetetramine, TFA = trifluoroacetic acid.

PCB without breaking the ester bond in its methylacrylate moiety. Furthermore, up to 6 hours of treatment with TFA did not destroy the ester backbone of PLGA (see the Supporting Information). The resistance of PLGA<sup>[29]</sup> and polylactic acid (PLA)<sup>[30–32]</sup> to acid degradation has been reported previously. Thus, PCB-*t*Bu can be used in a generic way for the synthesis of any amphiphilic block polymer containing one PCB block and a hydrophobic block, even if the hydrophobic part is subject to hydrolysis in water (e.g., PLA-PCB or PLGA-PCB).

To formulate PLGA-PCB NPs, we modified a solvent displacement method (or nanoprecipitation method)<sup>[19,33]</sup> in which the block copolymer is dissolved in a water-miscible organic solvent. Upon addition of water, in which the PCB block is soluble but the PLGA block is not, PLGA core/PCB shell structured NPs were formed. The organic solvent was evaporated while stirring, and left an aqueous solution in which the NPs hardened. When hydrophobic drugs are mixed with the copolymers in organic solvent, they become encapsulated in the hydrophobic core of the NP (Figure 1, Step 5). We found that the organic solvent can play a decisive role in NP formation owing to the sharp difference in polarity between the PLGA and PCB blocks. Single solvents, such as 2,2,2-trifluoroethanol (TFE), can dissolve the PLGA-PCB polymer largely because of the solubility of PLGA in this solvent; but this approach produced large particles with

heterogeneous size distributions ( $(515.8 \pm 50.0)$  nm,  $PDI = 0.527 \pm 0.056$ ). This outcome results from the microscopic insoluble state of PCB blocks in TFE. Thus, the formation of inverted PCB core/PLGA shell micelles does not favor small and homogeneous NPs. To improve the solubility of the PCB block in TFE, a TFE/MeOH (1:1) cosolvent system was used. As a result of using the cosolvent, NPs with a monodisperse size distribution were then fabricated in a reproducible way (e.g. NP size =  $(148.8 \pm 1.1)$  nm,  $PDI = 0.040 \pm 0.011$ ; Figure 2a). Note that TFE is preferred over dimethyl sulfoxide owing to the ease of evaporation of TFE upon stirring. For a typical formulation, PLGA-PCB copolymers self-assembled into NPs with very low PDI values. The yield was nearly quantitative, with no precipitates or micro-sized particles

formed during the assembly process. Thus, there was no need to use filtration to remove large particles. No surfactant was required during solvent displacement because the zwitterionic PCB shells stabilize the NPs in aqueous medium. Compared with PEGylated NP systems,<sup>[19]</sup> PCB has a better stabilization effect on NP dispersion because PCB is far more hydrophilic than PEG, thus less chain embedding by PLGA is expected during the self-assembly process. The sharp polarity difference between the copolymer blocks is responsible for such efficient assembly into small and homogeneous NPs. The zeta potential was measured for the PLGA-PCB NPs obtained above and was found to be  $(-43.5 \pm 1.0)$  mV. Meanwhile, PLGA NPs of the same size (NP size =  $(145.7 \pm$



**Figure 2.** Scanning electron microscopy (SEM) images for PLGA-PCB NPs before a) and after b) lyophilization and brief resuspension in water by using a pipette without sonication. The scale bar is 1  $\mu$ m. c) PLGA-PCB NP stability in PBS solutions of 10 wt% BSA and in 100% FBS at 37°C over 5 days. NP size (mean  $\pm$  SD,  $N=3$ ) is plotted as a function of time. NP stability upon high-speed centrifugation for d) PLGA-PCB NPs and e) PLGA-PCB/Dtxl NPs (1 wt% drug loading) was tested with three successive centrifugation cycles. After each centrifugation step (16110g, 15 min), supernatants were either removed or kept and the NP pellets were resuspended by using a pipette without sonication. f) Stability of PLGA NPs, PLGA-PCB NPs, and PLGA-PCB/Dtxl NPs with 1 wt% drug loading after lyophilization without any addition of a cryoprotectant. NP size (mean  $\pm$  SD,  $N=3$ ) is plotted and the polydispersity index values (PDI values, mean  $\pm$  SD,  $N=3$ ) accompanying each size point are indicated. SD = standard deviation.

3.9) nm, PDI =  $0.113 \pm 0.033$ ) had a zeta potential of ( $-68.1 \pm 1.8$ ) mV.

Docetaxel (Dtxl) was encapsulated into the PLGA-PCB NPs by the method described above, and the drug release profile was measured. For an initial drug input of 5 wt % in our typical formulation, the resulting PLGA-PCB/Dtxl NPs had about 1 wt % ( $(0.933 \pm 0.021)$  wt %) of drug payload, with a NP size of  $(138.5 \pm 0.6)$  nm, PDI of  $0.125 \pm 0.017$ , and zeta potential of ( $-34.8 \pm 1.3$ ) mV. Figure S1 (see the Supporting Information) shows that PCB-modified and unmodified PLGA NPs have similar sustained drug releasing profiles over 96 hours with 50 % of the encapsulated drug released in the first 8 hours. Drug release kinetics can be further tuned by changing the length of the PLGA blocks; the rate of drug release can be slowed by increasing the molecular weight of PLGA, as reported for the PLGA-PEG system.<sup>[11]</sup>

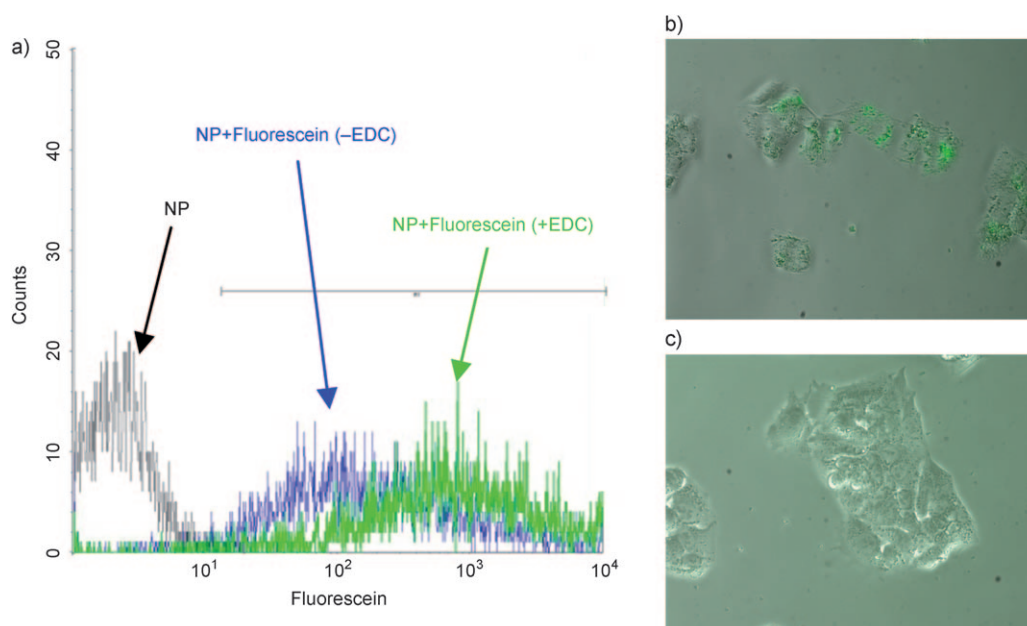
The stability of NPs in biologically relevant media such as serum determines their feasibility as drug-delivery vehicles for in vivo use. We hypothesize that PCB, which has been found to effectively resist nonspecific protein binding to surfaces in undiluted blood plasma and serum,<sup>[25–27]</sup> can stabilize hydrophobic PLGA NPs in complex media. In this work, we placed PLGA-PCB NPs in a phosphate buffered saline (PBS) solution of 10 wt % bovine serum albumin (BSA) or 100 % fetal bovine serum (FBS) solution at 37 °C, and measured the NP size as a function of time (see the Supporting Information, Figure S2). No size increase of PCB-modified NPs was observed during the 13-hour study, while unmodified PLGA NPs severely aggregated immediately after their immersion in these media. Furthermore, a long-term study of PLGA-PCB NPs showed that these particles maintain their original size over 5 days in both 10 wt % BSA and 100 % FBS media (Figure 2c). This result implies that PLGA-PCB NPs can potentially be used for in vivo drug delivery.

The stability of NPs upon postformulation or processing is also very important for NPs that are intended for clinical use. As PCB binds water through electrostatically induced hydration, it has stronger hydration than hydrogen-bonding materials such as PEG. It is expected that zwitterionic PCB polymers will protect NPs very well from the harsh conditions of various processing steps. High-speed centrifugation is widely used to form NP pellets for purification purposes, but PLGA-based NPs, including PEGylated NPs, tend to aggregate during pellet formation.<sup>[20]</sup> We found that PCB-modified PLGA NPs, with or without loaded drugs, were easily recovered from repeated high-speed centrifugation by brief resuspension of the pellets by using a pipette, thus eliminating the need for sonication. Pellets of unmodified PLGA NPs, once formed, however, cannot be resuspended without sonication. For PCB-modified NPs, when the supernatants were removed after each centrifugation step (as in standard NP purification procedures), then size increases of 6–9 % and 1–8 % were observed for PLGA-PCB NPs and PLGA-PCB/Dtxl NPs, respectively. These sizes were calculated from Figure 2d and e, respectively, and slight decreases in PDI values were also observed. The smaller PDI values obtained after each centrifugation cycle reflected the monodispersity of the remaining NPs. Note that the slight size increase after

each cycle was not caused by NP aggregation—smaller NPs are less likely to form the pellet during each centrifugation step, and thus the removal of supernatants will shift the size distribution of the recovered portion towards larger values. To further confirm the stability of the NPs, control experiments were performed in which the supernatant was not removed between centrifugation steps. It was found that the NPs maintained the same size and PDI value upon repeated centrifugation, thus indicating the ability of PCB to stabilize PLGA NPs (either with drug encapsulated or not) from aggregation upon pelleting. This ability is due to the strong hydration layer created by the PCB shells, which stabilize the hydrophobic PLGA cores from mechanically induced aggregation.

Freeze-drying is a necessary procedure for NP storage to prevent polymer degradation and drug leakage in aqueous storage media. To the best of our knowledge, no other polymer-based NPs can survive lyophilization without cryoprotectant additives.<sup>[19–21,33]</sup> Even for PEGylated NPs, additives such as 10 % sucrose<sup>[20,21]</sup> are required, as PEG is crystallized upon freeze-drying and thereby loses its ability to prevent NP aggregation.<sup>[19]</sup> The PCB-modified PLGA NPs reported here retain their stability after freeze-drying without any additives (Figure 2f). It is remarkable that a brief resuspension by using a pipette (no sonication needed) of the dry, PCB-modified NPs (either with or without loaded drug) recovered the NPs to the same mean diameter and low PDI values. After freeze-drying, these NPs were visualized by SEM images (Figure 2b). This remarkable behavior possibly results from strong PCB hydration and distinct PLGA/PCB blocks. Unlike PEG, PCB strongly binds a certain amount of water molecules to prevent crystallization during lyophilization. In addition, the efficient assembly of the copolymers, as a result of the sharp polarity contrast between the two blocks, renders an almost defect-free protective PCB shell, which keeps the hydrophobic cores apart even in a highly dehydrated environment. Because of their ability to survive in complex biological media and harsh postformulation processing, zwitterionic PCB-modified NPs uniquely address both industrial and clinical stability concerns.

Functionalization of NPs is required to attach biomolecules such as dyes and targeting ligands that are required for different purposes. To examine the feasibility of immobilizing those molecules of interest on PCB shells, we used fluorescein as a model ligand to generate fluorescent NPs. The carboxylate groups of PCB were converted into NHS esters in the presence of (denoted with a + sign in Figure 3) EDC and NHS and later conjugated with amine groups of the fluorescein molecules. To confirm that the conjugation was in fact successful on NP surfaces, we also undertook a negative control experiment; we prepared NPs without generating NHS esters in the absence of (denoted with a – sign in Figure 3) EDC but in the presence of NHS. Any binding of the dyes onto NPs in the negative control experiment should occur because of physical interactions. After treatment with fluorescein, NPs with NHS esters on the surface had significantly higher fluorescence intensity than the control. These experiments confirmed covalent coupling of the dyes to the NP surfaces (Figure 3a). The potential usefulness of



**Figure 3.** a) Flow cytometry study on PLGA-PCB NPs conjugated with  $\text{NH}_2$ -fluorescein. Bare NPs without fluorescein treatment, NPs treated with fluorescein (– EDC, + NHS), and NPs treated with fluorescein (+ EDC, + NHS) are shown in black, blue, and green curves, respectively. NPs covalently bound with fluorescein (green lines) show a very large increase of mean fluorescence intensity over NPs (– EDC, + NHS, + fluorescein). b) and c) Binding of PLGA-PCB/NBD NPs functionalized with galactose, to HepG2 cells. Cells were incubated for 2 h with PLGA-PCB/NBD NPs treated with b)  $\text{NH}_2$ -galactose (+ EDC, + NHS), and c)  $\text{NH}_2$ -galactose (– EDC, + NHS). Fluorescence image and phase contrast image were taken at 20 h and combined as illustrated in the figure.

PLGA-PCB NPs as targeted drug-delivery vehicles was also evaluated. A green fluorescent dye called NBD (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)) was used as a “visible” model drug and was encapsulated in the NPs. The resulting PLGA-PCB/NBD NPs were further conjugated with amine-modified galactose ligands with either in the presence of EDC and NHS, or in the absence of EDC but with NHS. These NP conjugates were incubated with HepG2 cells to test their cell binding abilities. Galactose is widely used to target asialoglycoprotein receptors in hepatoma cell lines (e.g. HepG2) *in vitro*<sup>[34–37]</sup> and hepatocytes *in vivo*.<sup>[34,35,38–40]</sup> We observed that PLGA-PCB/NBD NPs with immobilized galactose ligands (in the presence of EDC and NHS) readily bound to the cells, thus producing strong fluorescence as shown in Figure 3b. Meanwhile, PLGA-PCB/NBD NPs without the immobilized ligands (in the absence of EDC but with NHS) resulted in nontargeting and nonfouling NPs and had low cell-binding abilities (Figure 3c). These fluorescein and galactose/NBD results show that PCB-modified NPs can be easily functionalized with amine-terminated molecules which are useful for imaging and/or targeting purposes.

The structure of CB is similar to that of glycine betaine, which is a solute that is vital to osmotic regulation in living organisms.<sup>[41]</sup> Estimates of glycine betaine intake by humans are from 0.1 to 2.5 g day<sup>–1</sup>.<sup>[42]</sup> Thus modification of PLGA NPs with biomimetic PCB should not introduce any toxicity to FDA-approved PLGA. Indeed, a cytotoxicity assay (see the Supporting Information, Figure S3) showed that PLGA-PCB NPs were similar to PLGA NPs in terms of the viability of HepG2 cells after 24 hours of incubation at NP concentrations up to 10 mg mL<sup>–1</sup>. This concentration corresponds to

over 500 mg kg<sup>–1</sup> body weight for an adult human which is a much higher dose than required for *in vivo* drug delivery.

PCB-modified NPs are superior to PEGylated NPs because of their easy processing, extraordinary stability, and multifunctionality. The abundant carboxylate anions of PCB enable the attachment of targeting ligands, therapeutic drugs, and diagnostic labels all in one material through conventional NHS/EDC chemistry, thus making PCB a universal polymer for “theranostics”. The concept of using block copolymers with sharp polarity differences to achieve extraordinary stability in NPs, along with the idea of introducing superhydrophilic blocks to hydrophobic materials through hydrolyzable ester groups will guide the future design and synthesis of improved NPs for various applications.

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