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RAPID COMMUNICATION

Poly(carboxybetaine) nanomaterials enable long circulation and prevent polymer-specific antibody production

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Summary Poly(ethylene glycol) (PEG) has been incorporated into nanoparticles (NPs) to improve circulation time from systemic circulation for decades with limited success. Recent studies have shown that PEG induces the production of PEG-specific antibodies after repeated injections. Here, we demonstrate zwitterionic poly(carboxybetaine) (PCB)-based nanomaterials with no production of polymer-specific antibodies, while PEG-coated NPs generated PEG-specific antibodies. Furthermore, PCB-coated NPs exhibited prolonged circulation time and showed little change between the first and second doses ($t_{1/2}$ = 55.8 and 55.6 h), with no accelerated blood clearance suffered by PEG-coated NPs ($t_{1/2}$ drops from 8.7 to 5.2 h). These findings are significant advances toward solving the long-standing clinical challenge of developing nanomaterials that are able to resist both immune response and immediate bodily clearance.

Introduction

To date, numerous nanoparticle-based technologies quickly surged to the forefront of drug delivery, diagnostics, and many other areas [1]. However, the body has evolved finely tuned processes involving the innate and adaptive immune systems to remove non-self materials from the body. This makes engineering clinically relevant therapeutic nanoparticles an extreme challenge [2,3]. When nanoparticles (NPs) enter the bloodstream, they immediately encounter a complex environment of plasma proteins and leukocytes [2]. NP uptake by phagocytes may occur through various pathways [3,4] and can be facilitated by the adsorption of plasma proteins (opsonins) to the particle surface [2]. The ideal NP platform is one whose integrity is undisturbed, providing extended systemic circulation to maximize delivery to the target site. Additionally, it should be nontoxic and invisible to the immune system. Various strategies have been studied

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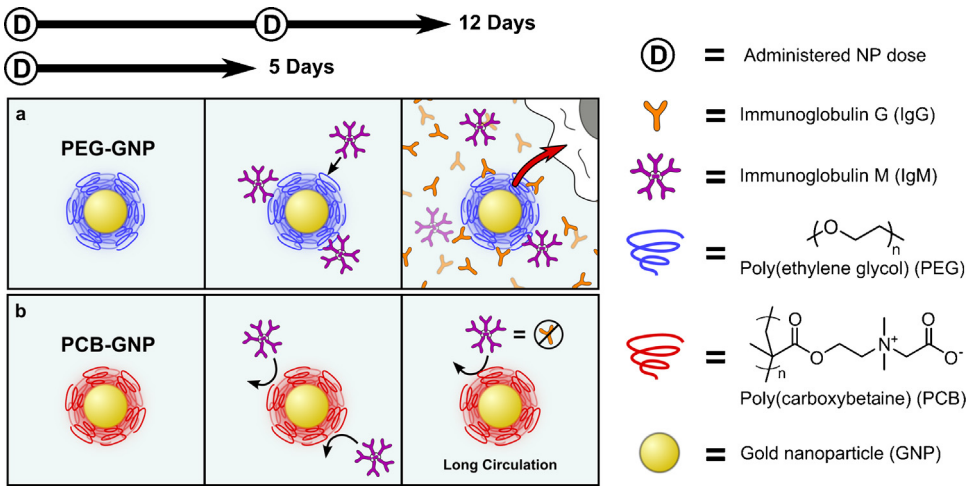

Figure 1 Schematic illustrations of the sequence of events after PEG-GNPs and PCB-GNPs enter the blood stream. (a) PEG-GNPs. Anti-PEG IgM is produced over 5 days in response to PEG-GNPs. IgG is also produced in response to additional PEG-GNPs injections as the result of previous IgM response. (b) PCB-GNPs. Extremely low fouling stealth PCB coating effectively shields NPs from nonspecific opsonization and immune response, leading to long-circulating NPs with zero antibody production. Without IgM stimulation, IgG is also not produced.

for immunosuppression, including manipulating NP physical properties (*e.g.*, size/architecture) or surface chemistry (*e.g.*, PEGylation) [2,5—7]. Although the modification with PEG can reduce nonspecific protein adsorption, preventing flocculation, opsonization, and subsequent complement activation [8], it was already shown in a toxicological study of PEG in 1950 that PEG has the propensity to induce blood clotting and clumping of cells, leading to embolism formation [9]. More recently, discovery of a PEG-specific immune response has triggered further investigation [9—12]. It was suggested that PEGylated NPs also induce production of anti-PEG immunoglobulin M (IgM) antibodies. IgM antibodies stimulate the complement system and lead to the clearance of the NPs [9,11,12] (Fig. 1a). Additionally, previous reports have shown a markedly higher occurrence (22—25%) of anti-PEG antibodies in the healthy blood donor population [13]. Thus, it is important to keep in mind that the generation of PEG-specific antibodies will affect the efficacy of PEG-based therapeutics.

Various attributes of NPs need to be tailored for effective drug delivery applications, including circulation time, targeting, cellular internalization, and selective drug release [14,15]. Among these, the ability of NPs to remain in circulation is one of the most important determinants of therapeutic effect [15]. Longer circulating NPs are more likely to reach their target, allowing high therapeutic efficacy and/or imaging sensitivity, as well as reduced accumulation in normal tissues and avoiding side effects [3]. However, most NPs are susceptible to opsonization, resulting in the accelerated recognition and subsequent clearance by the mononuclear phagocyte system (MPS) [16]. Although numerous strategies involving PEG coatings have been applied to improve the circulation length of NPs, success has been limited to circulation times of 8 to 10 h [15]. Previously, zwitterionic materials have been shown to have high resistance to nonspecific protein adsorption even in undiluted blood plasma and serum [17,18]. These zwitterionic materials bind to water stronger than PEG *via* electrostatically induced hydration, where PEG interacts with water *via* hydrogen-binding [19]. It has been shown that superhydrophilic poly(carboxybetaine) (PCB) is superior to PEG at maintaining protein stability and bioactivity [17,20]. Thus, it is conceivable that zwitterionic materials can shield NPs from nonspecific binding and thus prevent the production of antibody more effectively, thereby improving their circulation time. In this work, we hypothesize that NPs protected by ultra-low fouling and superhydrophilic zwitterionic PCB materials will have excellent stealth properties, thereby preventing bodily clearance (Fig. 1b). To test this, gold nanoparticles (GNPs) were used as a model system, which also act as a label for detection. In order to investigate only the effect of the polymer coatings, the particle size and surface charge of the NPs are kept the same.

Materials and methods

Preparation of PEG-GNPs and PCB-GNPs

PEG-GNPs were synthesized according to a previously published method [21]. PCB-GNPs were prepared *via* surface initiated atom transfer radical polymerization (SI-ATRP). In a typical reaction, 74 mg Cu(I)Br and 1.8 g carboxybetaine-1-methacrylate tert-butyl ester (CBMA-1-tBu) monomer were placed into a Schlenk tube and subjected to three vacuum-nitrogen cycles. Then, 7 mL of degassed dimethylformamide (DMF) was added and the content was purged with N_2 before 148.6 mg 1,1,4,7,10,10-hexamethyltriethylenetetramine was added to the flask *via* syringe. The reaction mixture was thoroughly purged with N_2 for an additional 15 min to yield solution A. Similarly, initiator (11-mercaptoundecyl 2-bromoisobutyrate, $Br(CH_3)_2COO(CH_2)_{11}SH$) coated GNPs in DMF were deoxygenated by bubbling N_2 for 15 min to yield solution B. Polymerization was started by transferring solution A into solution B under N_2. After reaction was stirred at 750 rpm

and 50 °C for 24 h, the PCB-GNPs were precipitated in ethyl ether and collected by centrifugation. Hydrolysis was carried out by dissolving in acidic water (pH 3.0) overnight. Then the product was dispersed in deionized water, and other impurities including residual monomers, initiators, and catalysts were removed by using a 100 kDa molecular weight cutoff Amicon Ultra centrifugal filter. The polymer-modified GNPs were collected by centrifugation and redispersed in phosphate-buffered saline (PBS) before further use, yielding a dark red solution.

Animal studies

The *in vivo* antibody production, circulation time, and biodistribution of GNPs were studied using Spraque Dawley rats (body weight ~150 g) as the animal model. Each GNP sample was studies in six duplicates to generate statistical significance. All animal experiments adhered to federal guidelines and were approved by the University of Washington Animal Care and Use Committee. Two groups of rats were formed. For Group 1 rats, which received one injection, the GNPs (PEG-GNPs or PCB-GNPs) were administered *via* the tail vein at a dose of 1 mg/kg body weight under anesthesia and sterile treatment. Control animals received PBS instead of GNPs. To study the *in vivo* circulation time, at 5 min, 4 h, 8 h, 24 h, and 48 h after the injection, a 50 μL blood sample was collected, digested, and analyzed by the elementary analysis method, inductively coupled plasma atomic emission spectroscopy (ICP-AES, Elan DRC-e, PerkinElmer). On the fifth day after the injection, 5 mL of blood were drawn by using cardiac punch method and serum was prepared for IgM measurement. Biodistribution was assessed after the animals were euthanized by CO_2 inhalation, where organs (heart, liver, spleen, lung, and kidneys) were collected, digested in aqua regia, and analyzed by ICP-AES.

For Group 2 rats, which received two injections, PEG-GNPs or PCB-GNPs were administered intravenously at a dose of 1 mg/kg body weight 7 days apart. Control animals received PBS instead of GNPs for both injections. On the fifth day after the second injection, 5 mL of blood were drawn for immunoglobulin G (IgG) measurement. The *in vivo* circulation time and biodistribution tests were performed the same as in Group 1.

Determination of IgM and IgG levels with enzyme-linked immunosorbent assay (ELISA)

Using the 5 mL blood samples drawn as described in the previous section, serum samples were obtained by incubation for 30 min at room temperature followed by centrifugation at 3000 rpm at 4 °C for 15 min. Quantification of IgM and IgG levels were performed using the Rat IgM ELISA Quantitation Kit and the Rat IgG ELISA Quantitation Kit from BETHYL Laboratories, TX, USA. Briefly, 100 μL of 1:100 diluted primary antibody (Goat anti-Rat IgM or Goat anti-Rat IgG antibody) in coating buffer (50 mM carbonate-bicarbonate, pH 9.6) was added to each well of a 96-well plate and then incubated at room temperature for 1 h. The wells were then washed five times with wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0). Then, 200 μL of blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) was added to each well and incubated 30 min at room temperature. After incubation, the blocking solution was aspirated and the wells were washed five times with wash solution and 100 μL of 1:100 standard or sample in the sample diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) was added to the wells. After incubation for 1 h at room temperature, the blocking solution was removed and the wells were washed five times with the wash solution. 100 μL of secondary antibody conjugated to horseradish peroxidase (HRP) for detection (Goat anti-Rat IgM-HRP or Goat anti-Rat IgG-HRP conjugate) in the conjugate diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH8.0) was transferred to each well. After incubation for 1 h at room temperature, the detection antibody was removed and the wells were washed five times with the wash solution. The enzyme assay was initiated by adding 3,3′,5,5′-Tetramethylbenzidine substrate solution. After a 15 min incubation, the reaction was stopped by adding 100 μL of 0.18 M H_2SO_4 and the absorbance was measured at 450 nm using a microplate reader.

Determination of IgM levels in serum incubated with GNPs

125 μL PEG-GNPs or PCB-GNPs (1 mg/mL) was incubated in 500 μL of serum derived from rats administered with PEG-GNPs at 37 °C for 15 min. GNPs was separated out of serum by centrifugation (13,200 rpm for 30 min). The IgM level of the supernatant was directly measured by ELISA as described above. The centrifuged aliquot was re-dispersed in PBS and centrifuged for two more times to remove unbound IgM. The final centrifuged pellet was dispersed in diluent solution (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) and the IgM level was measured by ELISA.

Results and discussion

GNPs characterization

Size is an important parameter in the design of long-circulating NPs. The size should be large enough to avoid renal clearance [22], but not too large as to induce opsonization and MPS clearance [23]. Thus, comparisons are only useful between NP formulations of similar sizes. PCB-GNPs were made by performing SI-ATRP of the GNPs. Since the ATRP initiator is hydrophobic while the CB monomer is hydrophilic, the hydrophobic CBMA-1-tBu monomer was chosen to graft well-controlled polymer brushes with high packing densities on GNPs in an organic solvent. Upon subsequent acid hydrolysis of the protected monomer, densely coated PCB-GNPs were achieved (Fig. 1b). The average hydrodynamic diameter was 40.2 nm, measured by dynamic light scattering (DLS) based on intensity. Similar to the size of PCB-GNPs, the average hydrodynamic diameter of the corresponding PEG-GNPs is 39.8 nm.

It has been shown previously that the particles bearing a cationic or anionic surface charge can attract more phagocytes than neutral particles of the same size [4]. Thus, neutral PCB-GNPs and PEG-GNPs are preferable. To demonstrate this, PCB-GNPs and PEG-GNPs were suspended in PBS and monitored by DLS for surface zeta potential at different pH values. Zeta potential was found to be neutral for both

PCB-GNPs (−0.09 mV) and PEG-GNPs (−0.08 mV) at pH 7.4 (Supplementary Fig. S1a). Moreover, GNPs coated with PEG and PCB were further studied in undiluted human plasma at 37 °C. This was done by measuring the change in particle size over time using DLS. For PEG-GNPs, the diameter increased to 300 nm after the incubation period, indicating instability through protein adsorption and particulate aggregation (Supplementary Fig. S1b). The adsorption of proteins can increase the effective size of NPs, change their effective surface charges, and increase phagocyte uptake [2]. Meanwhile, the PCB-GNPs remained the same size over the incubation period, showing protection against aggregation afforded by the PCB coating. Since the opsonization of NPs leads to their accelerated recognition, phagocytosis, and elimination, PCB-GNPs with no plasma opsonin adsorption will evade immune detection [6,15].

In vivo circulation evaluation

We then investigated the *in vivo* circulation half-life of the PEG-GNPs and PCB-GNPs after repeated injections. In a clinical setting, repeated injections are often necessary, such as in the case of multiple courses of chemotherapy [11]. However, PEG suffers from the accelerated blood clearance (ABC) phenomenon. Dams et al. first reported that for multiple administrations of PEG liposomes in rats, the second dose showed a drastically reduced blood liposome content when compared to that of the first dose, from 52.6 ± 3.7 to 0.6 ± 0.1% [24]. This was evidence of the ABC phenomenon for PEG. There has been growing evidence showing that the ABC phenomenon not only affects the bioavailability of drugs, but targeting is also decreased [11].

Fig. 2 shows that the PCB-coated GNPs had superior blood retention compared to the PEG-functionalized GNPs. At 48 h after the first dose, PCB-GNPs exhibited 50% overall retention, as compared to 3% exhibited by the PEG-GNPs. Using a two-compartment model, commonly used in previous studies [25], the elimination half-life was calculated as 243.9 h for PCB-GNPs and 10.7 h for the PEG-coated GNPs after the first dose (Fig. 2a, Supplementary Fig. S2a, and Table S1). After the second dose, the elimination half-life was 268.0 h and 8.2 h for PCB-GNPs and PEG-GNPs, respectively (Fig. 2b, Supplementary Fig. S2b, and Table S1). Using a one-compartment model, the blood half-lives of PCB-GNPs and PEG-GNPs were 55.8 and 8.7 h after the first dose (Supplementary Fig. S2c). This is comparable to previous results of 40-nm PEG-coated GNPs in a rat model, where the particles possessed *in vivo* circulation half-life of 6.0 h [26]. Previous *in vivo* pharmacokinetic studies of poly(poly(ethylene glycol) methyl ether methacrylate)-coated silicone dioxide NPs *via* SI-ATRP showed that the optimal blood half-life was around 20 h with a well-controlled core size and chain length [27]. Thus, the PCB-GNPs in this work possess long-circulating properties.

From the data, PEG-GNPs were seen to stimulate the ABC phenomenon: the first dose of PEG-GNPs ($t_{1/2}$ = 8.7 h) accelerated the clearance of the second dose ($t_{1/2}$ = 5.2 h) given one week later. In contrast, the one-compartment circulation time of PCB-GNPs showed little change from $t_{1/2}$ = 55.8 h to $t_{1/2}$ = 55.6 h (Supplementary Fig. S2c and Fig. S2d). A same trend was observed when the two-compartment model was

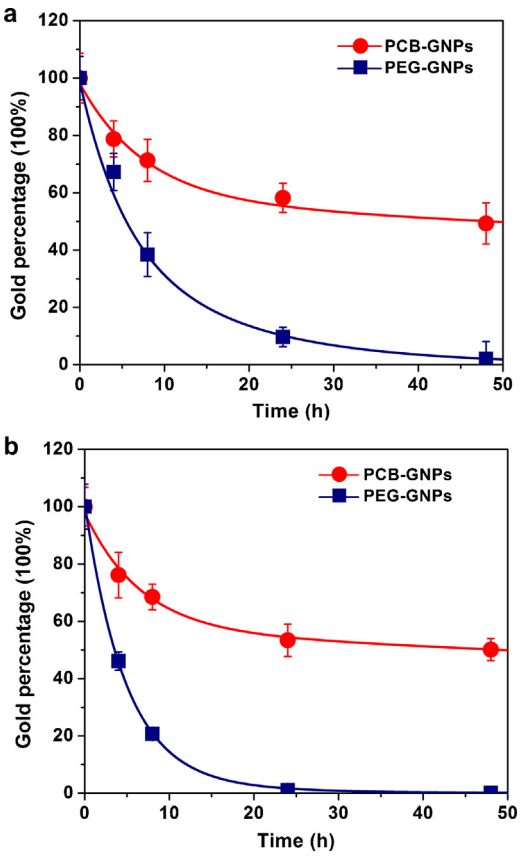

Figure 2 *In vivo* circulation time of the GNPs after the first and second administrations. (a) Blood clearance profile of the first dose of PCB-coated GNPs (red circles) and PEG-coated GNPs (blue squares). (b) Blood clearance profile of the second dose of PCB-coated GNPs (red circles) and PEG-coated GNPs (blue squares). The GNPs were injected intravenously through the tail vein of rats at a dose of 1 mg/kg body weight. At various time points, blood samples were taken from tail nick and measured for gold concentration with ICP-AES to evaluate the systemic circulation lifetime of the NPs. Each value is averaged from 6 rats. Standard deviations are shown as error bars.

applied (Supplementary Fig. S2a and Fig. S2b). These results show how PCB coatings are able to provide long circulation times to NPs and also prevent induction of the ABC phenomenon (Fig. 1b). We believe the stealth characteristics of PCB come from mimicking the ionic natural chemistry found on the surface of proteins [20]. Previous studies have also shown that by using mechanobiological mimicry of red blood cells (RBCs), elimination half-life was improved [28]. In another study, RBC membrane-camouflaged polymeric NPs were used to extend circulating time [10].

Quantification of antibody production

Since it was suggested that the production of anti-PEG antibodies is responsible for the ABC phenomenon [11], we measured IgM and IgG levels after the first and second injections, respectively. Previous research studied IgM production as a function of time after injection and found that the IgM level reached peaked after 5 days [29]. Another study also

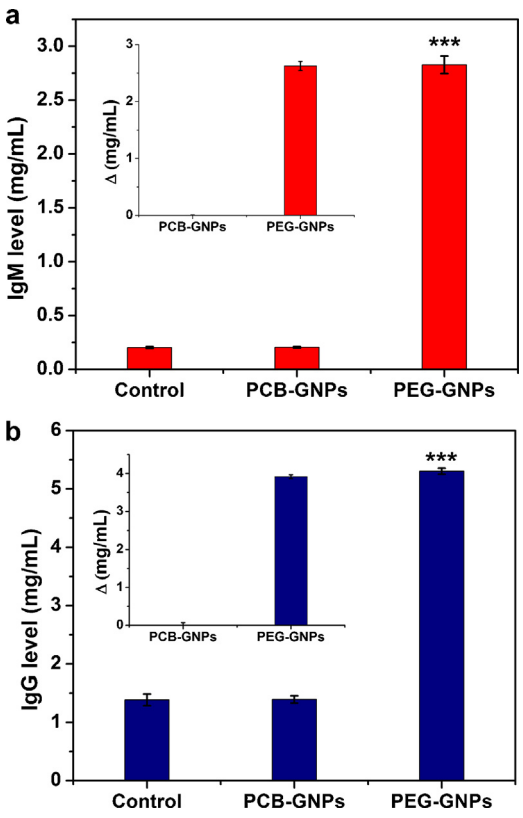

Figure 3 IgM and IgG levels following one and two intravenous injections of PBS or GNPs in rats. (a) IgM levels after the first injection; (b) IgG levels after the second injection. The insets are plots when the antibody level of control group with PBS is subtracted. 100 μL of each GNP sample was administered into the rat *via* tail vein injection. Control animals received PBS instead of GNPs. Blood was taken on the fifth day after the first and second injections for IgM and IgG measurements, respectively. Blood sampling from control group was used to establish baseline antibody level for each rat. IgM (red bar) and IgG (blue bar) were detected using ELISA. Each value represents the mean ± S.D. of six animals from each sample. *P* values apply to differences against the control with $P < 0.05$ being considered as statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed unpaired Student's *t* test.

showed that the IgG level continues to increase with additional injections [30]. IgG production, which is dependent on IgM stimulation, is a secondary and more effective immune response to foreign materials in the body. Based on these studies, we tested IgM levels 5 days after a single injection (Group 1), and IgG levels 12 days after two injections (Group 2), with a second injection administered 7 days after the first injection. These results are summarized in Fig. 3. Blood sampling from control groups were used to establish baseline antibody levels. IgM levels in rats that received PEG-GNPs were 13.9 times higher than baseline levels. The same trend was seen for IgG, which was 3.8-fold higher than the baseline. In contrast, the effect of administering PCB-GNPs showed no noticeable increase in either IgM or IgG. As shown in the insets of Fig. 3, the changes in level of IgM and IgG from PCB-GNPs were almost zero after subtracting baseline levels. These results show how PCB coatings, through

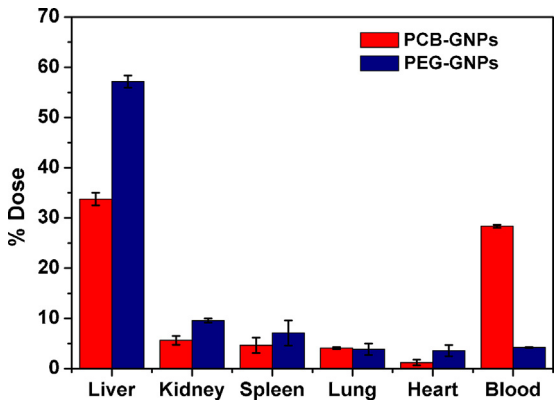

Figure 4 Biodistribution of the PEG-GNPs and PCB-GNPs after the first injection. GNPs were injected intravenously into the rats. On the fifth day after the first injection, the organs from all groups of rats were collected, digested in aqua regia, and analyzed by ICP-AES. GNP accumulation in each organ is normalized to the total dose after the first injections. Each value is averaged from 6 rats. Standard deviations are shown as error bars.

their stealth properties, can avoid stimulation of the adaptive immune system, observed by the lack of IgM and IgG antibody productions.

Biodistribution of GNPs

To further investigate how the GNPs were cleared from circulation, biodistribution studies were performed. Fig. 4 shows the GNPs accumulation in each organ as a percent of the total administered dose 5 days after a single injection (Group 1). It can be observed that the majority the NPs were either found in the blood or the liver. The majority of the PEG-GNPs were found in the liver, as is often observed with NPs. While some PCB-GNPs were also found in the liver, a large portion was still found in the blood. Group 2, after euthanasia, was also evaluated for biodistribution, but this involved two injections over a 12-day period. This data can be seen in Supplementary Fig. S3 as a percent of the sum of both doses. Similar to Group 1, there was a significant amount of PCB-GNPs still in the blood, but this time no detectable PEG-GNPs. The ABC phenomenon was observed by the absence of PEG-GNPs in the blood after the second injection. This is the most compelling evidence showing the clinical potential of PCB coatings for injectable, long circulating NPs. This data is also represented in gold content per gram of organ (Supplementary Fig. S4).

Determination of serum proteins associated with GNPs

Finally, to determine whether IgM generated in rats administered with PEG-GNPs specifically binds to PEG, PEG-GNPs and PCB-GNPs were incubated separately in serum derived from these rats and levels of IgM bound to each of GNPs were measured (Fig. 5). When serum incubated with PEG-GNPs was centrifuged to pellet the GNPs, some IgM is present in the supernatant, representing the nonspecific IgM not bound

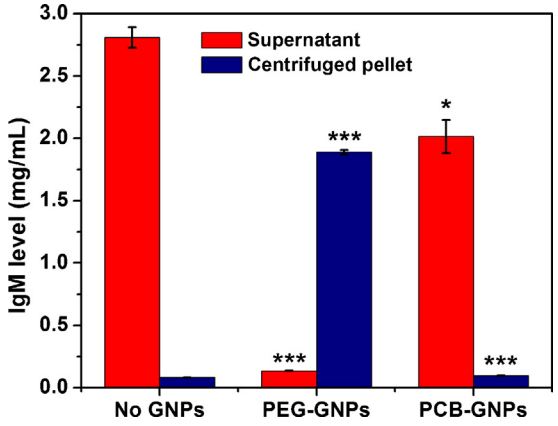

Figure 5 IgM levels in serum incubated with GNPs. PEG-GNPs or PCB-GNPs were incubated in serum collected from rats administered with PEG-GNPs to allow IgM association and binding. They were separated from serum by centrifugation and IgM levels in the supernatant or the pellets containing GNPs were determined by ELISA. What appears to be missing between the total antibody concentration of the no-GNP control and the PEG-GNP and PCB-GNP tests would be loss due to repeated centrifugation steps not done in the control. Each value represents the mean ± S.D. of three separate samples. *P* values apply to differences from serum with no GNP incubation with $P < 0.05$ being considered as statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, two-tailed unpaired Student's *t* test.

to PEG-GNPs and a larger amount is present in the pellet due to binding with PEG-GNPs prior to centrifugation. On the other hand, when serum incubated with PCB-GNPs was centrifuged, the majority of IgM antibody remains in the supernatants with some present in the pellet but not at a significant level. What appears to be missing between the total antibody concentration between the no-GNP control and the PEG-GNP and PCB-GNP tests would be loss due to repeated centrifugation steps not done in the control. This supports the idea that the elevated IgM present in test rats subjected to PEG-GNPs was induced by PEG-GNPs and selectively binds to PEG.

Conclusions

In summary, we demonstrated that zwitterionic nanomaterials can prevent the production of polymer-specific antibodies while maintaining long circulation, even after repeated injections. Our findings emphasize the importance of extremely low fouling [5], stealth PCB coatings with high packing densities for avoiding the ABC phenomenon. It should be noted that avoiding this mechanism of adaptive clearance is a major hurdle for practical use of nanomedicine in a clinical setting. The majority of reported injectable NPs rarely address this issue due to its difficulty. This PCB surface material exhibits superior *in vivo* performance over its PEG counterpart, including extremely low antibody response, along with prolonged residence time in blood, thus demonstrating its great potential as a robust coating for drug delivery and diagnostic platforms.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nantod.2014.02.004.

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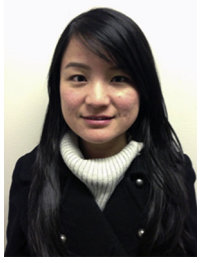
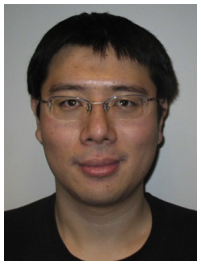


Sijun Liu is currently a graduate student under Prof. Shaoyi Jiang's supervision in the Department of Bioengineering at the University of Washington. She received her B.S. in Chemical Biology and B.A. in Molecular and Cell Biology (immunology) from the University of California, Berkeley. Her current research focuses on the development of zwitterionic biomaterials in the context of nanomedicine and translational science.

Tao Bai is currently pursuing his Ph.D. degree in the Department of Chemical Engineering at the University of Washington, Seattle under the guidance of Prof. Shaoyi Jiang. He received his B.Eng. degree in polymer materials from Tianjin University, China in 2011. His current research is focused on the development of zwitterionic materials for biological applications.

Andrew J. Keefe recently received his Ph.D. in Chemical Engineering from the University of Washington, studying biomaterials under Professor Shaoyi Jiang. His work focused on stabilizing biomaterials and biologics with zwitterionic polymers. He received his B.S. in biological and chemical engineering from Rensselaer Polytechnic Institute. He is currently a research scientist at Shire Pharmaceuticals.



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Shaoyi Jiang is the Boeing-Roundhill Professor of Chemical Engineering and Adjunct Professor of Bioengineering at the University of Washington, Seattle. He received his Ph.D. degree in chemical engineering from Cornell University in 1993. He was a postdoctoral fellow at the University of California, Berkeley between 1993 and 1994 and a research fellow at California Institute of Technology between 1994 and 1996 both in chemistry. Currently, he serves as a Senior Editor for Langmuir. He is a Fellow of the American Institute of Chemical Engineering, a Fellow of the American Institute of Medical and Biological Engineering and a Member of the Washington State Academy of Sciences. His current research focuses on the molecular understanding, design and development of zwitterionic-based functional materials for biomedical and engineering applications.