Nanoparticles are widely used as imaging probes\[^{[1]}\] or as drug carriers,\[^{[2]}\] however, the design rules to guide their properties for in vivo application remain incomplete. Until recently, the physical characteristics of nanoparticles such as size, shape, hydrophobicity, and net charge have been mostly considered as primary handles to achieve nanoparticles with desired in vivo characteristics. However, we hypothesized that the detailed arrangement of ligands also has a strong influence on the in vivo behavior of nanoparticles, because the interactions of nanoparticles with the surrounding biomolecules or cellular structures are mediated by surface-to-surface contacts. In support of this hypothesis, previous work has shown that specific arrangements of hydrophobic ligands on the nanoparticle surface can affect the cellular uptake pathway of the particles.\[^{[3]}\] Here, we demonstrate the influence of spatial distribution of surface charge on the binding and transport behavior of nanoparticles in vivo. An understanding of the influence of charge distribution on nanoparticle behavior is essential, as most nanoparticle coatings employ charged functional groups for further conjugation with biomolecules or drugs. Even though there have been reports on nanoparticles employing uncharged functional groups for conjugations,\[^{[4]}\] most widely used conjugation methods still rely on ester bond formation, amide coupling, or maleimide–thiol coupling and utilize carboxylic acid or amine groups. Furthermore, studying the influence of surface charge arrangement on nanoparticle behavior inspires the design of zwitterionic surface coatings, which were recently introduced as possible alternative for bulky nonionic polymeric surface coatings such as poly(ethylene glycol) (PEG).\[^{[5]}\]

For this study, quantum dots (QDs) coated with newly designed betaine-functionalized poly(imidazole) ligands (BPILs) were utilized. The unique optical properties of QDs\[^{[6]}\] allow facile analysis of QD behavior by using optical microscopes, rather than elemental analysis,\[^{[7]}\] magnetic resonance imaging,\[^{[8]}\] or electron microscopy,\[^{[9]}\] and simultaneous analysis of multiple targets. Recent advances in the development of nonionic or charged surface coatings enabled preparation of high-quality QDs in aqueous buffers.\[^{[10]}\] However, most zwitterionic surface coatings for QDs that are currently reported employ thiol as a binding group\[^{[5a,b,11]}\] and thus suffer from poor stability in ambient condition owing to the oxidation of thiols and significant nonspecific binding to serum proteins.\[^{[10c,11]}\]

To prepare stable and biocompatible zwitterionic QDs, we designed new zwitterionic coatings containing multiple imidazole moieties, which are chemically stable, as cooperative binding groups to the QD surface. In addition to an enhanced solution stability, the new zwitterionic QDs have other potential advantages such as a compact size, a narrow polydispersity index of the polymer, straightforward modulation of charge density and charge distribution, and minimal nonspecific binding towards cells and proteins.

BPILs, random copolymers employing imidazole groups for QD binding and sulfobetaine or carboxybetaine groups for water solubilization, are synthesized in three steps (Scheme 1): 1) RAFT-mediated polymerization of the backbone (3), 2) betainization of the polymers (4, 6), and 3) cleavage of tert-butyloxycarbonyl (Boc) protecting groups (5, 7). RAFT-mediated polymerization allows tight control over the mean and standard deviation of the resulting polymer weights (Figure S5 in the Supporting Information). Postmodification of a polymerized backbone by using highly strained 1,3-propane sultone and β-propiolactone enables one-step conversion of tertiary amines to sulfobetaine or carboxybetaine by releasing the ring strain. The net charge and degree of freely exposed amines of BPILs were controlled by tuning the conversion efficiencies of tertiary amines to betaine moieties.
Water-soluble QDs coated with the BPILs (BPIL QDs) were prepared by exchanging the native hydrophobic ligands with BPILs. Various types of QDs emitting from the near infrared to the visible were successfully brought into water with both sulfobetaine-functionalized poly(imidazole) ligands (SBPILs) and carboxybetaine-functionalized poly(imidazole) ligands (CBPILs): InAs/CdZnS emitting at 750 nm (QD750), CdSe/CdS emitting at 570 nm (QD570), and CdSe/CdZnS emitting at 612 nm (QD612). Owing to multidentate imidazole binding units, BPIL QDs exhibit greatly enhanced stability across a wide pH range (Figure S10 in the Supporting Information). In addition, both CBPIL and SBPIL QD solutions were stable under ambient conditions for over a month, which is a significant enhancement over the hours to days shelf lives of reported zwitterionic QDs. 

Scheme 1. Scheme for synthesis of sulfobetaine- and carboxybetaine-functionalized poly(imidazole) ligands (SBPILs and CBPILs). Random copolymer backbones (3) are synthesized through radical addition fragmentation chain transfer (RAFT)-mediated polymerization and betainized using 1,3-propanesultone and β-propiolactone to yield SBPILs (5) and CBPILs (7), respectively. AIBN = azobisobutyronitrile.

Figure 1. Surface net charge measurements. a) Zeta potential measurements illustrate that SBPIL QDs are slightly negative and CBPIL QDs are neutral. Zeta potential measurements for DHLA QDs (QDs coated with dihydrolipoic acid, highly negative) and PEGPIL QDs (QDs coated with poly(ethylene glycol)-functionalized PILs, nonionic) are shown as a reference. b) Gel electrophoresis of the PEGPIL QDs (first lane), SBPIL QDs (second lane), CBPIL QDs (third lane), and DHLA QDs (fourth lane) confirm the zeta potential measurement.

QDs were prepared by using SBPIL80% (80% conversion efficiency; see Table S1 in the Supporting Information).

To verify the influence of spatial charge configuration on QD–cell interactions in vitro, nonspecific binding of zwitterionic QDs (SBPIL QDs and CBPIL QDs) with cultured HeLa cells was evaluated. Previous reports\(^ \text{[12]} \) predict neutral and hydrophilic QDs (SBPIL40% QDs and CBPIL100% QDs) to exhibit minimal cell binding. Instead, cells incubated with SBPIL80% QDs and CBPIL100% QDs displayed a significant increase of fluorescence signal, thus suggesting nonspecific binding of the QDs to cells (Figure 2a,b and Figure S13 in the Supporting Information). In addition, dramatic differences in nonspecific cell binding were observed depending on the betainization efficiencies of the SBPILs (Figure 2a).

The results with SBPIL QDs illustrate that, even for QDs that are neutral or slightly charged, the number of spatially exposed amines, which are positively charged groups, is an important factor that determines the level of nonspecific binding to cells. SBPIL80% QDs possessing nonconverted tertiary amines exhibited significant nonspecific binding to cells despite the zero net charge of the particle, while SBPIL100% QDs with no exposed amines showed virtually no binding to cells despite their small negative charge.

In vitro nonspecific binding results with CBPIL QDs reinforce the importance of the absence of positively charged groups on the outermost layer to achieve minimal nonspecific cell adsorption. Even though the chemical structure of CBPIL100% indicates that the tertiary amine groups are fully converted to carboxybetaine moieties and thereby well-screened by negative carboxylate groups, quaternary amines are more likely to be in the outermost layer (exposed) than carboxylate groups owing to the affinity of carboxylate to the OD surface. The binding affinity of carboxylic acids to the OD surface is similar to that of imidazole groups, which are anchoring groups in BPILs. Therefore, both carboxylic acids and imidazole moieties are expected to bind to the OD surface. Attraction of carboxylate groups towards the OD surface has been previously confirmed. The high level of exposed amines resulted in significant nonspecific binding for CBPIL100% QDs despite their nearly neutral charge (zeta...
potential of $-0.3 \text{ mV}$; Figure 2b). CBPIL\textsubscript{100\%} QDs also exhibited strong interaction with diverse polymeric beads such as sephadex, superpose, sephacryl, and superfine. In contrast, the quaternary amines on SBPIL\textsubscript{100\%} QDs are well-screened from the environment by the sulfonates, as sulfonate groups do not interact with the QD surface. Minimal non-specific binding was observed for SBPIL\textsubscript{100\%} QDs.

Previous studies on gold surfaces (both nanoparticles and flat substrates) coated with carboxybetaine ligands\cite{15} also confirm our observation that spatially exposed positive charges trigger the high level of nonspecific binding. Unlike for CBPIL\textsubscript{100\%} QDs, carboxybetaine ligands are anchored to the gold substrates through thiols. Since the binding affinity of thiols to gold is far greater than that of carboxylate groups,\cite{16} the entire carboxybetaine moieties are exposed to the environment, that is, positive amine groups are well-screened by negative carboxylate groups. Therefore, gold substrates coated with carboxybetaine ligands are reported to exhibit extremely low non-specific binding properties to cells and proteins.\cite{5e,15b,17}

The influence of the relative distribution of charges on nonspecific binding of nanoparticles in vivo was evaluated by injecting SBPIL\textsubscript{100\%} QDs and CBPIL\textsubscript{100\%} QDs intravenously into mice and monitoring the vessels over time. Upon systemic administration, SBPIL\textsubscript{100\%} QDs, which are likely to display negative charges on their outermost layer, cleared from vessels without leaving any evidence of nonspecific accumulation. In contrast, CBPIL\textsubscript{100\%} QDs, which are likely to display positive charge in their outermost layer, accumulate on vessels nonspecifically (Figure 2c,d). Electrostatic interactions between the exposed positive charges on CBPIL QDs and negatively charged groups presented on the surface of endothelial cells may trigger non-specific accumulation of CBPIL\textsubscript{100\%} QDs.

Furthermore, we compared the in vivo binding and transport behavior of zwitterionic QDs versus nonionic QDs to study the influence of the existence of charge in neutral nanoparticles. SBPIL\textsubscript{100\%} QDs were chosen to represent zwitterionic QDs owing to their low nonspecific binding properties, and PEGPIL QDs (QDs coated with poly(ethylene glycol)-functionalized PILs) were selected as an example of nonionic QDs. Previously reported PEGPILs\cite{10c} have the same polymer structure as BPILs except the water-solubilizing groups and therefore serve as an ideal control pair for BPIL QDs.
In vitro nonspecific binding tests show that zwitterionic QDs exhibit slightly higher nonspecific binding to cells and serum proteins. SBPIL100% QDs exhibited slightly higher nonspecific cell adsorption than PEGPIL QDs at concentrations above 100 nM (bottom). In contrast, the level of nonspecific binding for PEGPIL QDs remains minimal up to 200 nM (top). The intensity scale bars shown on the left and right correspond to the left and right parts of the images, respectively. The vertical lines in the right parts of the images indicate where the intensity profile shown below was measured. Refer to Figure S12 in the Supporting Information for the images replotted by using a heatmap for better visualization of the QD signal. b) Comparison of clearance kinetics. Clearance kinetics of SBPIL QD612 and PEGPIL QD570 (left panel), which have very similar hydrodynamic diameters, and SBPIL QD750 and PEGPIL QD750 (right panel), that were synthesized with the same QD cores. Both results illustrate that SBPIL QDs clear from the vessels much faster than PEGPIL QDs. c) Biodistribution (%ID/g tissue; ID = injected dose) of zwitterionic QDs 24 h after QD injection shown for different organs and the tumor. SBPIL QDs are cleared through liver and spleen, which may indicate RES clearance (RES = reticuloendothelial system). Abbreviations used are: Hrt, heart; Intstn, intestine; Kdn, kidney; Lvr, liver; Lng, lung; Spln, spleen; and Tmr, tumor. Error bars represent 95% confidence interval.

Lastly, tumor transport measurements showed that nonionic QDs extravasate from vessels and diffuse into tumor at an order of magnitude faster than zwitterionic QDs displaying negatively charged groups in their outermost layer. Transvascular transport of QDs in tumors was assessed by measuring effective permeability of the particles in two different types of breast tumors: E0771 and MCaIV. The effective permeability measures the ability of QDs to extravasate from vessels and diffuse into the extravascular space, and is independent of clearance rate. Of the two breast tumor types, MCaIV has exceptionally large pores. The average pore cutoff size of MCaIV is 1.2 to 2 μm, whereas most tumors show a pore cutoff size of 380 to 780 nm. In both nonionic PEG chains are more effective than compact and zwitterionic, i.e., ionic betaine groups at preventing biomolecules from sticking nonspecifically to the surface better.

Analysis of the clearance kinetics of zwitterionic QDs and nonionic QDs shows that zwitterionic QDs clear from blood significantly faster than nonionic QDs. When zwitterionic QDs (SBPIL100% QD612) and nonionic QDs (PEGPIL QD750) with similar net charge (–3.1 mV for PEGPIL QDs, –13.1 mV for SBPIL100% QDs) and hydrodynamic sizes (ca. 10 nm, Figure S16 in the Supporting Information) were co-injected into mice, zwitterionic QDs cleared an order of magnitude faster than nonionic QDs. In both Figures 3a and Figure S17 in the Supporting Information).
tumor models, the zwitterionic QDs exhibited an effective permeability that was one to two orders of magnitude lower than nonionic QDs (Figure 4). The unexpectedly low effective permeability that was one to two orders of magnitude lower than nonionic QDs (Figure 4). The unexpectedly low effective permeability that was one to two orders of magnitude lower than nonionic QDs (Figure 4). The unexpectedly low effective permeability that was one to two orders of magnitude lower than nonionic QDs (Figure 4). The unexpectedly low effective permeability that was one to two orders of magnitude lower than nonionic QDs (Figure 4).

Figure 4. Comparison of transvascular transport of zwitterionic QDs versus nonionic QDs in breast cancer. a) Real-time intravital microscopy of ligand-dependent nanoparticle distribution in a murine breast tumor. A mixture of SBPIL QDs (left) and PEGPIL QDs (right), which has the same hydrodynamic diameter, was injected intravenously into an SCID mouse bearing an E0771 murine mammary adenocarcinoma in a mammary fat pad chamber. Angiographic images demonstrate distribution of the nanoparticles one hour after injection. The initial fluorescence was recorded shortly after injection, and is depicted as the gray line. The images show that SBPIL QDs extravasate from vessels significantly less than PEGPIL QDs. Images are maximum intensity projections of 3D volumes. Scale bar = 100 μm. b) Effective permeability of SBPIL QD60 and PEGPIL QD60 in mice bearing an E0771 murine mammary adenocarcinoma (left) and a MCAV murine mammary adenocarcinoma (right). Solid bars represent the values obtained with SBPIL QD60. Lined bars represent the values obtained with PEGPIL QD60. Error bars represent 95% confidence interval. In both tumor models, SBPIL QD60 always showed significantly smaller effective permeability than PEGPIL QD60.

In this study, we designed and synthesized a new class of zwitterionic QD ligands to prepare neutral QDs with modulated spatial charge arrangements. Greatly enhanced stability and the low biolinking of BPIL QDs allowed an in-depth analysis of in vivo behavior. Our study demonstrates the critical importance of spatial charge arrangements in determining in vivo characteristics of nanoparticles. Zwitterionic QDs displaying positive charges in their outermost layer exhibit significant nonspecific accumulation to cultured cells and vessels in live mice, whereas zwitterionic QDs displaying negative charges in their outermost layer show minimal nonspecific adsorption. In addition, comparison of in vivo behavior between zwitterionic QDs and nonionic QDs illustrate that exposed charged groups cause an enhanced interaction of zwitterionic QDs with their environment and result in significantly different binding and transport behavior in vivo. Therefore, when designing nanoparticles for in vivo applications, the three-dimensional configuration of charges needs to be closely considered with specific applications in mind. For instance, to minimize nonspecific interaction of nanoparticles with their environment, positively charged groups need to be spatially screened. In addition, the development of new conjugation methods employing neutral functional groups is highly recommended for achieving nanoparticles with long blood circulation and rapid transvascular delivery. These findings can be extended to other substrates, inorganic particles, and polymeric nanoparticles that can be used as imaging agents or drug carriers.


