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Zwitterionic Polymer Ligands: an Ideal Surface Coating to Totally Suppress Protein-Nanoparticle Corona Formation?

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KEYWORDS: nanoparticle, protein corona, zwitterion.

ABSTRACT

In the last few years, zwitterionic polymers have been developed as antifouling surface coatings. However, their ability to completely suppress protein adsorption at the surface of nanoparticles in complex biological media remains undemonstrated. Here we investigate the formation of hard (irreversible) and soft (reversible) protein corona around model nanoparticles (NPs) coated with sulfobetaine (SB), phosphorylcholine (PC) and carboxybetaine (CB) polymer ligands in model albumin solutions and in whole serum. We show for the first time a complete absence of protein corona around SB-coated NPs, while PC- and CB-coated NPs undergo reversible adsorption or partial aggregation. These dramatic differences cannot be described by naïve hard/soft acid/base electrostatic interactions. Single NP tracking in the cytoplasm of live cells corroborate these in vitro observations. Finally, while modification of SB polymers with additional charged groups lead to consequent protein adsorption, addition of small neutral targeting moieties preserves antifouling and enable efficient intracellular targeting.

INTRODUCTION

Nanoparticles (NPs) exposed to biological fluids rapidly adsorb proteins.[1] These form the so-called “hard” and “soft” corona according to their binding strength and exchange rates at the NP surface. The composition of this protein corona depends on many parameters, including particle size, surface chemistry (hydrophilicity, surface charge, etc.), environmental conditions (time, temperature, etc.) and the nature and composition of biological fluids. Recent studies suggested that the protein corona may depend on the patient health and may be used for diagnostics.[2,3] However, in many in vitro or in vivo applications, the protein corona dictates the fate of nanocarriers and induces fast recognition by the immune system. It therefore reduces the circulation lifetime in the blood stream, as well as the targeting efficiency, and influences cellular uptake, biodistribution and toxicity.[4–10] PEGylation (PEG = Poly-ethylene glycol) is commonly used to reduce non-specific protein adsorption in order to increase the blood half-lives and facilitate specific targeting and drug delivery. Yet, even though PEGylation has been proven to efficiently reduce protein adsorption, it is unable to completely suppress it.[7,11–15] Moreover, PEG is not biodegradable and in vivo accumulation can cause undesirable side effects.[16] In addition, contrarily to initial assumptions, PEG may actually elicit immune responses that limit the targeting of PEG-conjugated agents in patients.[16] More recently, other hydrophilic polymers have been proposed as an alternative to PEG such as polysaccharides and polybetaines. The latter belong to the zwitterionic family and have been used as solubilizing ligands for a variety of nanoparticles such as silica[17–20], gold[21–30], quantum dots (QDs)[31–46], SPION[30,47–51] and others.[52–58] Zwitterionic moieties consist of positively (quaternary ammonium in the case of betaines) and negatively charged groups linked by a small carbon chain. In consequence, polyzwitterions present an overall neutral charge. The super-hydrophilic nature of polyzwitterions, due to the strong hydration of the charged groups, is usually evoked to explain their antifouling performances. In addition, several studies suggest a lower immunogenicity of zwitterion-encapsulated compared to PEG-modified proteins.[59,60]

Yet, in contrast to PEGylated nanoparticles, nonspecific interactions between zwitterion-coated nanoparticles and biomolecules are still poorly characterized, which hinders further biomedical applications. Recent fluorescence correlation spectroscopy (FCS) pointed to a negligible adsorption of the model human serum albumin onto quantum dots (QDs) coated with monomeric sulfobetaine based ligands.[37] However there have been only a few studies in more complex whole serum[24] and none in cytoplasmic environments. Finally, while the nature of the zwitterion anionic charge strongly influences hydration,[61,62] its impact on protein adsorption remain poorly understood.

In the present work, we analyze the formation of hard and soft corona around nanoparticles coated with three betaine zwitterionic polymers: phosphorylcholine, carboxybetaine and sulfobetaine, differing in their anionic moieties. In addition, we investigate the effect of incorporating functional reactive groups for further bioconjugation on nanoparticle-protein interactions. As model inorganic particles, we used semiconductor quantum dots since their fluorescence enable ultrasensitive detection, even in complex

media. We employed different complementary characterization of (i) hard and soft protein corona by colorimetric assays and FCS, respectively, upon incubation with albumin or whole serum and (ii) single QD diffusion dynamics in the cytoplasm of living cells, which are extremely sensitive even to transient nonspecific interactions. Remarkably, these in vitro and in vivo complementary assays yield a consistent picture. In particular, we show for the first time that polysulfobetaine enable a virtually complete suppression of both hard and soft corona and unhindered intracellular Brownian motion. In comparison, polyphosphorylcholine and carboxybetaine are less efficient at preventing nanoparticle-protein interactions in these highly concentrated media. Modifications of sulfobetaine ligands by addition of small neutral functional group such as biotin preserve antifouling, in contrast to the addition of extra charges.

RESULTS AND DISCUSSION

The nanoparticles used in this study were fluorescent core/multishell CdSe/CdS/ZnS quantum dots (~7 nm in diameter) and coated with zwitterionic polymer ligands. The polymers were synthesized by RAFT using methacrylamide (or methacrylate) monomers bearing terminal sulfobetaine (SB), phosphorylcholine (PC) or carboxybetaine (CB) functions, with Mn comprised between 3,000 and 20,000 g.mol⁻¹ and a polydispersity index IP lower than 1.12. Then a vinylimidazole block (degree of polymerization ~ 10) was introduced at the end of the polyzwitterionic chains to ensure binding of the ligand onto the inorganic QD surface (Figure 1, S1 – S8).[33]

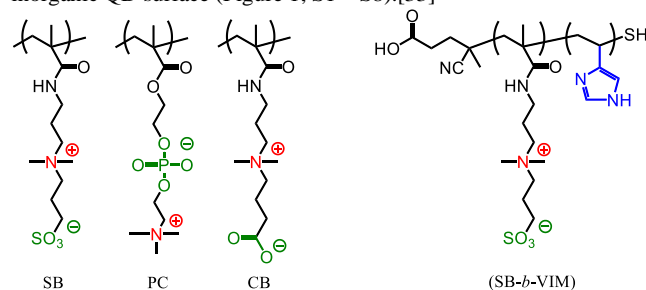


Figure 1. Sulfobetaine (SB), phosphorylcholine (PC) and carboxybetaine (CB) polyzwitterions used in this study. Diblock sulfobetaine-vinylimidazole copolymer (SB-b-VIM).

Compared to statistical polymers, this block architecture should prevent exposition of anchoring functions to the external environment which could induce nonspecific interactions. The details of polymer and QD synthesis, ligand exchange procedure and purification steps are reported in the Supporting Information. Quantum dots coated with these polymeric ligands remain colloidal stable for more than 1 year when stored at 4°C. Diffusion Ordered Spectroscopy (DOSY) NMR measurements show the absence of free polymers, which demonstrates that purification was efficient and that polymeric ligands do not desorb from the QD surface (Figure S10 and S11). Zetametry measurements show that all zwitterionic QDs are nearly neutral at physiological pH. Their zeta potentials do not significantly differ

QD + Polymers	Mn (g.mol ⁻¹)	IP	Zeta potential (mV)	Rh (nm)	Number of polymer chains / QD	Surface coverage (mg.m ⁻²)
QD-SB	3 400	1.12		5.6 ± 0.5	27.5	1.0 ± 0.3
QD-SB	6 900	1.07	-10 ± 3.4 mV	6.3 ± 0.3	18.5	1.4 ± 0.3
QD-CBM	4 500	1.08	-4.7 ± 2.5 mV	6.5 ± 0.3	21.5	1.0 ± 0.3
QD-PC	8 200	1.07	-6.7 ± 2.8 mV	7.25 ± 0.4	13.25	1.2 ± 0.2
QD-SB-NH ₃ ⁺	11 800	1.10	-4.9 ± 5.3 mV	6.85 ± 0.3	11.25	1.2 ± 0.2

Table 1. Characterization of QDs coated with zwitterionic polymer ligands: polymer number molecular weight (Mn), polydispersity index IP, zeta potential, hydrodynamic radius from FCS, average number of polymer chains grafted per QD and surface coverage density.

from each other (Table 1). Quantification of the number of polymer ligands on the surface of QDs was performed using ^1H NMR (Figure S12, Table 1). Interestingly, the polymeric surface density remains constant at around $1 \text{ mg} \cdot \text{m}^{-2}$, independently of the nature and size of the zwitterionic block, in the range of $3,400\text{--}11,800 \text{ g} \cdot \text{mol}^{-1}$. As we will see with the following experiments, within this range, the size of the zwitterionic polymer does not seem to influence protein adsorption either.

In order to investigate the stealth properties of different zwitterionic surface chemistries, we evaluated the adsorption of proteins at the surface of QDs after incubation in whole human serum (HS) or with increasing concentrations of bovine serum albumin (BSA), a commonly used model protein present at millimolar concentrations in the serum. QDs ($1 \mu\text{M}$) were incubated with BSA (1 mM) or HS for 1 h and then isolated from free and loosely bound proteins by several ultracentrifugation cycles. The tightly bound proteins forming the hard corona were quantified by fluorescamine assay after dissolution of the inorganic QDs in acidic solution. After calibration with known BSA concentrations, this assay provides an average number of BSA proteins per QD, or BSA equivalent in the case of HS incubation (1 BSA eq. corresponding to the adsorption of $\sim 66,000 \text{ g}$ protein per mol of QD).

In addition, protein adsorption was investigated by FCS. The advantage of this technique is its ability to characterize protein-nanoparticle interaction in concentrated protein solutions. This yields information on the total protein corona, composed of both tightly and loosely bound proteins. FCS provides the diffusion coefficient of QDs with a good sensitivity and variations of hydrodynamic radii due to protein adsorption may be determined with nanometric precision. The measurements were conducted at a QD concentration of 50 nM in the presence of 0 to $1100 \mu\text{M}$ of BSA or of undiluted HS (see Supporting Information, Figure S14-S16).

Nonspecific interactions of polyzwitterion coated QDs.

First, we present the antifouling behavior of QDs functionalized with sulfobetaine polymer. This polyzwitterionic ligand is able to virtually eliminate hard corona formation with BSA and serum proteins (see Figure 2a). Indeed, the fluorescamine assays did not reveal differences in fluorescence signal between QDs incubated in protein-free and protein-containing solutions. The presumed number of proteins within the hard corona was then estimated below the detection limit ($< 0.05 \text{ BSA eq. per QD}$). The absence of protein adsorption was also highlighted using FCS experiments. No variation of the hydrodynamic QD radius was noticed in presence of HS or BSA even at the highest (1.1 mM) concentration. Moreover, the replacement of methacrylamide by methacrylate sulfobetaine based monomers for the polymer

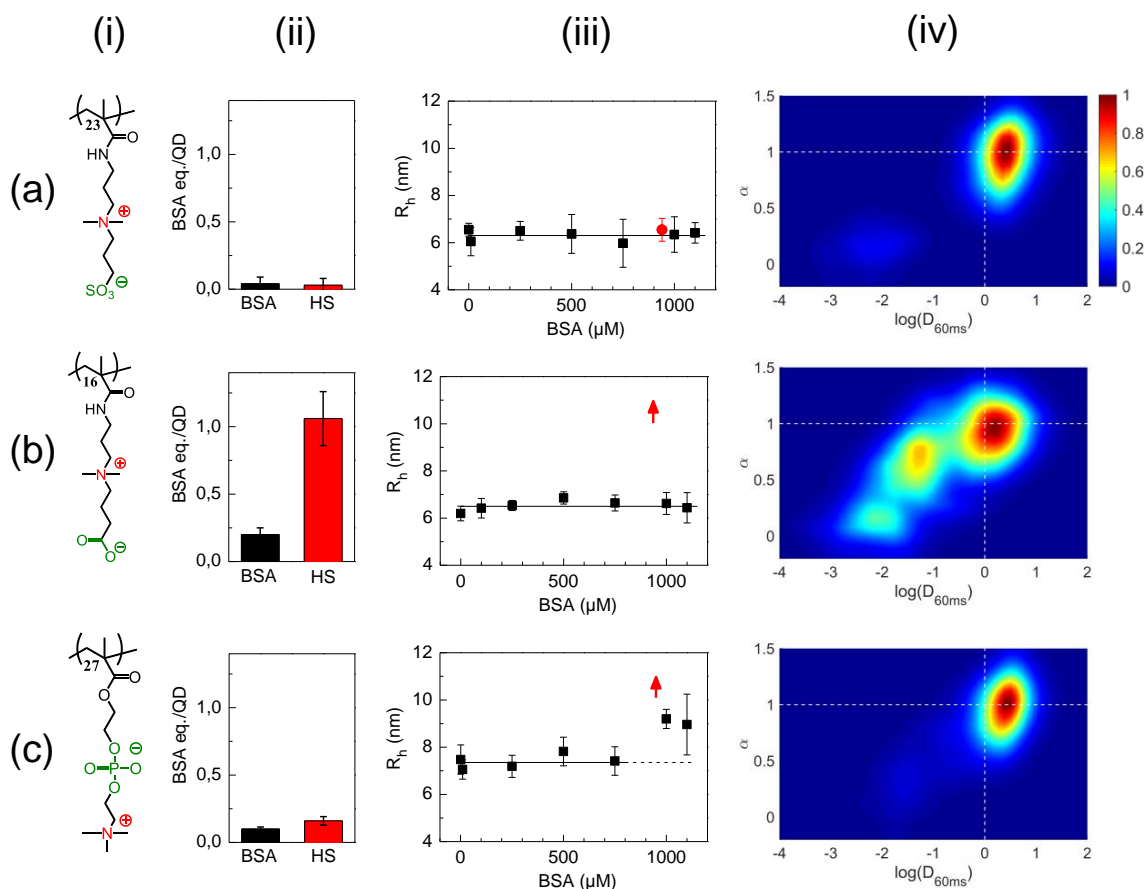


Figure 2. Influence of polyzwitterions grafted on the surface of QDs on protein adsorption and cytoplasmic diffusion. From left to right, (i) structure of the sulfobetaine (a), carboxybetaine (b) and phosphorylcholine (c) zwitterions. (ii) Average number of BSA equivalent adsorbed per QD within the hard corona. (iii) QD hydrodynamic radius determined by FCS as a function of BSA (black squares) concentration and whole HS (red circle, placed at $950 \mu\text{M}$ equivalent BSA concentration). Red arrows reflect an aggregation of the QDs (iv) Density plot of mobility measurements in the α - $\log(D_{60\text{ms}})$ plane. Colour code represents the local density of trajectories normalized between 0 (blue) and 1 (red). $\alpha = 1$ for purely Brownian motion and $\alpha < 1$ for sub-diffusive behavior. White dashed lines indicating the conditions for Brownian motion are a guide to the eye.

synthesis does not affect the antifouling properties (Figure S17). The variety of the nature and arrangement of positively and negatively charged groups in zwitterion moieties enables tuning of charge densities to optimize solubility and antifouling properties. Carboxybetaine (CB) and phosphorylcholine (PC) are examples of classical zwitterionic head groups and seem to have antifouling properties comparable to SB. Polymers with these two zwitterion motifs have been synthesized and tested against protein adsorption. CB monomers are usually prepared by ring opening alkylation of tertiary amine by propiolactone producing 3-ammoniopropionate.[38][63] However, this CB moiety is prone to facile Hoffman elimination limiting the usefulness of polyzwitterion bearing this motif.[64] To overcome this difficulty, CB monomer with 3 intermediate carbons between quaternary ammonium and carboxyl group have been synthesized here by reacting N-[3-(dimethylamino)propyl] methacrylamide with methyl 4-bromobutyrate followed by the hydrolysis of the ester functionality (Figure S2).[65] For this polyzwitterion, the antifouling behavior towards BSA is slightly less efficient than with SB (see Figure 2b). There is no variation of the QD radius during mixing with BSA, but we show that the concentration of residual albumin forming hard corona is slightly higher than the detection limit (ca. 0.2 BSA per QD). The main differences between SB and CB ligands appear with serum. When CB-coated QDs are mixed with HS, we observe intense events in the emission intensity time trace during FCS measurements which reveals formation of large aggregates (Figure S18). These aggregates can be partially removed after centrifugation (20,000 g; 5 min). They immediately reappear after an extra addition of serum solution, but not after addition of a fresh QD solution (Figures S19). This suggests that HS contains a fraction of proteins present in small amounts and different from albumin that induce aggregation of some of the CB-coated nanoparticles. These aggregates do not dissociate during ultracentrifugation washing steps, which translates into a high hard protein corona in HS (ca. 1 BSA eq./QD).

Phosphorylcholine ligand still has a different behavior from the other polyzwitterions (Figure 2c). FCS experiments show an increase of the hydrodynamic radius of the PC-coated QDs due to protein adsorption at concentration higher than 1 mM. The overall radius increase (~2 nm) is consistent with the formation of a small protein layer without any aggregation. In addition, the negligible level of residual protein measured after washing highlights the “dynamic” nature of BSA-PC interactions. A weak aggregation tendency is evidenced by FCS, when the PC-capped QDs are exposed to serum (Figure S18). However, as for BSA, the adsorbed proteins are easily removed during washing steps.

Interestingly, no influence of polymer size on the soft corona in BSA or HS was detected when these experiments were conducted with much shorter SB polymer ($M_n = 3,400 \text{ g}\cdot\text{mol}^{-1}$) or longer PC polymer ($M_n = 18,600 \text{ g}\cdot\text{mol}^{-1}$), as presented in Figure 3. This suggests once again that interactions of zwitterionic QDs depend on the nature of the zwitterion and not primarily on the size of the polymer ligand, within the size range tested here. Finally, no significant increase in hydrodynamic radii of SB-coated QDs was observed after incubation in BSA or HS for a few days at 37 °C (Figure 4). This shows that the zwitterionic QD retain their antifouling behaviour over prolonged periods of time in complex biological environments.

Combining the characterization of “hard” and “dynamic” protein-nanoparticle interactions enables classifying the polyzwitterions according to their interaction strength with proteins present in the blood. PC exhibits an intermediate behavior between SB, which totally suppresses nonspecific binding, and CB, which form strong aggregates with certain blood proteins, but not with albumin. All these polyzwitterions belong to the betaine family and differ in the chemical nature of their negatively charged sites. They can be ordered following their charge density as carboxylate > phosphate > sulfonate.[66] Following Collins’s concept based on Hofmeister’s pioneering work, ion pairing should be favored between ions of opposite charge and similar charge density (hard/hard or soft/soft interactions).[67,68] In contrast, soft/hard interactions are less probable. Such simple concept can be evoked to explain some properties of polyzwitterions, such as solubility in aqueous salt solution.[69,70] If one were to consider zwitterions as simple additions of independent positive and negative charges, one should expect stronger interactions of cationic amino acids,

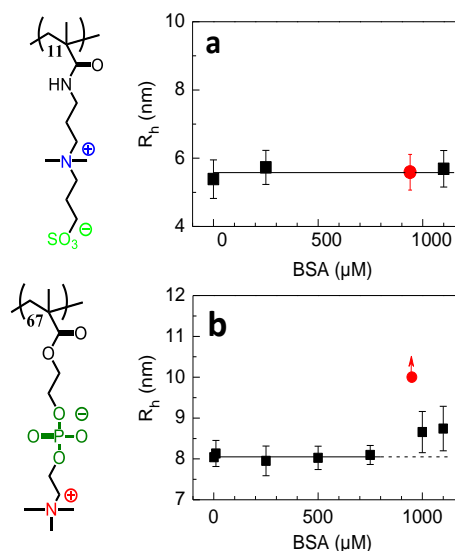


Figure 3. FCS hydrodynamic radius as a function of BSA (black squares) concentration and whole HS (red circle or arrow when QDs aggregate) for QDs capped with (a) SB ($M_n = 3,400 \text{ g}\cdot\text{mol}^{-1}$) or (b) PC ($M_n = 18,600 \text{ g}\cdot\text{mol}^{-1}$) polymer ligands.

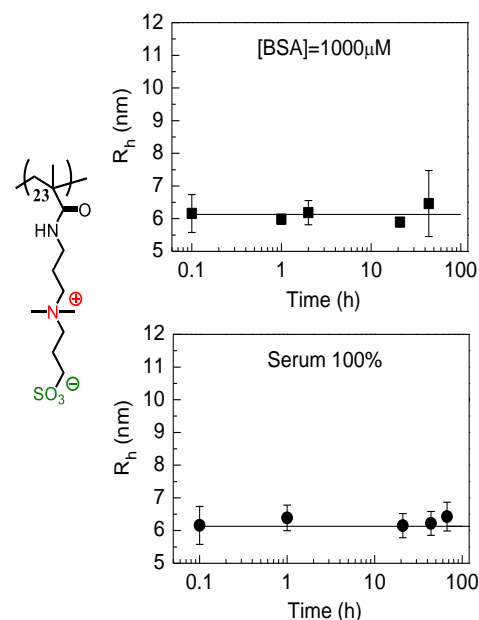


Figure 4. Hydrodynamic radius of SB ($M_n = 6,900 \text{ g}\cdot\text{mol}^{-1}$) coated QDs as a function of time after incubation in BSA (1000 μM , top) and HS (bottom) at 37°C.

which are considered as soft, with sulfobetaine than with carboxybetaine.[71] The present results invalidate this prediction, which suggests that interactions between polyzwitterions and proteins cannot be described within a simple charge interaction model. On the other hand, molecular dynamic studies have shown that the number of water molecules in the first hydration shell of the anion follows the order: sulfobetaine > phosphorylcholine > carboxybetaine.[61,72] This stronger hydration of sulfobetaine may possibly explain its superior antifouling property. Interestingly, in comparison, we observed that QDs coated with a poly(poly(ethylene glycol)-*b*-vinylimidazole) copolymer ligand of similar architecture (first PEG block: $M_n = 7500 \text{ g}\cdot\text{mol}^{-1}$, $M_w/M_n = 1.1$) showed similar properties in terms of ligand surface density and hydrodynamic diameter in saline buffer (Table S1), but failed to completely prevent non specific adsorption of albumin above 250 μM as well as in whole serum (Figure S20). Since the antifouling efficiencies of PEG surface coatings depend on PEG lengths and surface densities,[11] a more systematic study would be needed to definitely conclude on the comparison of zwitterionic and PEG surface chemistries.

We then examine the behavior of these polyelectrolyte-coated nanoparticles in the even more complex intracellular environment. Nonspecific interactions of nanoparticles with cytoplasmic components is a key determinant for their intracellular dynamics as they lead to sub-diffusive, instead of Brownian motion and reduced mobility.[73] QDs were internalized in the cytoplasm of HeLa cells using electroporation (see Supporting Information) resulting in 10-100 pM intracellular concentrations. The mean square displacements of individual QD trajectories were analyzed to estimate their instantaneous diffusion coefficients (calculated over the first 60 ms) as well as the associated anomalous exponents α ($\alpha = 1$ for a purely Brownian motion and $\alpha < 1$ for a sub-diffusive behavior), as shown in Figure 2 (iv) and Table 2. The results are consistent with the in vitro observations above: SB- and PC-coated QDs have a quasi-Brownian motion ($\langle\alpha\rangle = 0.89$ and 0.92 , respectively), consistent with their excellent antifouling behavior. Their instantaneous diffusion coefficients are high ($\langle D\rangle = 2.68$ and $2.57 \mu\text{m}^2\cdot\text{s}^{-1}$ for SB- and PC-coated QDs, respectively). This is more than 1 order of magnitude faster than commercial PEGylated QDs or latex nanoparticles, as measured in a previous study.[73] and consistent with very weak, if any, nonspecific interactions with cytoplasmic components. On the other hand, CB-coated QDs show slightly lower diffusion coefficients ($\langle D\rangle = 0.98 \mu\text{m}^2\cdot\text{s}^{-1}$) and anomalous exponents ($\langle\alpha\rangle = 0.73$). In particular, a non-negligible population fraction of QD trajectories (31 %) had an exponent $\langle\alpha\rangle \sim 0.6$, indicative of strong interactions with cytoplasmic components. QDs introduced in the cell cytoplasm using micro-injection instead of electroporation showed the same behavior (data not shown).

Table 2. Mean and standard error (SEM) of diffusion coefficients and α for each sample. N is the number of analyzed trajectories.

Influence of extra chemical functions inserted in SB polyelectrolyte.

Targeting nanoparticles towards specific biomolecules requires their functionalization with targeting moieties. SB and PC polyelectrolytes are chemically inert, so additional reactive chemical functions must be inserted into these polymers. For CB, the carboxylate function was used directly for conjugation of amine-containing (bio)molecule.[74] In this case, each amide

group formed by conjugation modifies the charge balance by suppressing a carboxylate and leaving a remaining quaternary ammonium cation, which, as we will see, may reduce antifouling properties. All modifications of polyelectrolytes are susceptible to introduce interactions between the added chemical functions and proteins via electrostatic, hydrogen or hydrophobic bonds. To investigate these effects, we modified the pure SB block by adding a small proportion (ca. 15%) of primary amine terminated methacrylamide monomers (APMA) during polymerization (see Supporting Information). The average number of APMA per polymer chain was estimated to 4. Reactions with a large excess of NHS-ester activated organic dyes indicate that about 20 amine functions per QD are accessible for chemical modifications. These primary amines are protonated ($-\text{NH}_3^+$) at physiological pH, which increases slightly the QD zeta potential from about -10.0 ± 3.4 mV for pure SB coated QDs to -4.9 ± 3.0 mV. This is also confirmed by the difference of the gel electrophoretic mobility as reported in Figure S21. The addition of positive charges in the globally neutral zwitterionic coating induces dramatic changes in the nanoparticle-protein interactions. FCS experiments now reveal adsorption of a protein monolayer when QDs are incubated with full serum and BSA at a concentration higher than $500 \mu\text{M}$ (see Figure 5a and Figure S22). After washing cycles, a low amount of residual proteins remains tightly bound to the QD surface.

In a second set of experiments, cationic terminal amines were transformed into anionic carboxylates after reacting QDs with an excess of bis(3-sulfo-N-hydroxysuccinimide) ester (BS3) following by hydrolysis of the remaining NHS esters. Replacement of cationic by anionic functions reduces interactions with proteins, but does not completely suppress them (Figure 5b). The minimum concentration of BSA needed to induce adsorption is twice as large, while no increase in hydrodynamic size was observed in whole HS. This demonstrates that even a small modification of the charge balance in the initially neutral polyelectrolyte polymer has a strong impact on nanoparticle-protein interaction. The effect is much more pronounced for positive charges because proteins are mostly negatively charged. Functionalization of the cationic amine groups with a neutral biotin leads to a full recovery of the antifouling properties of the

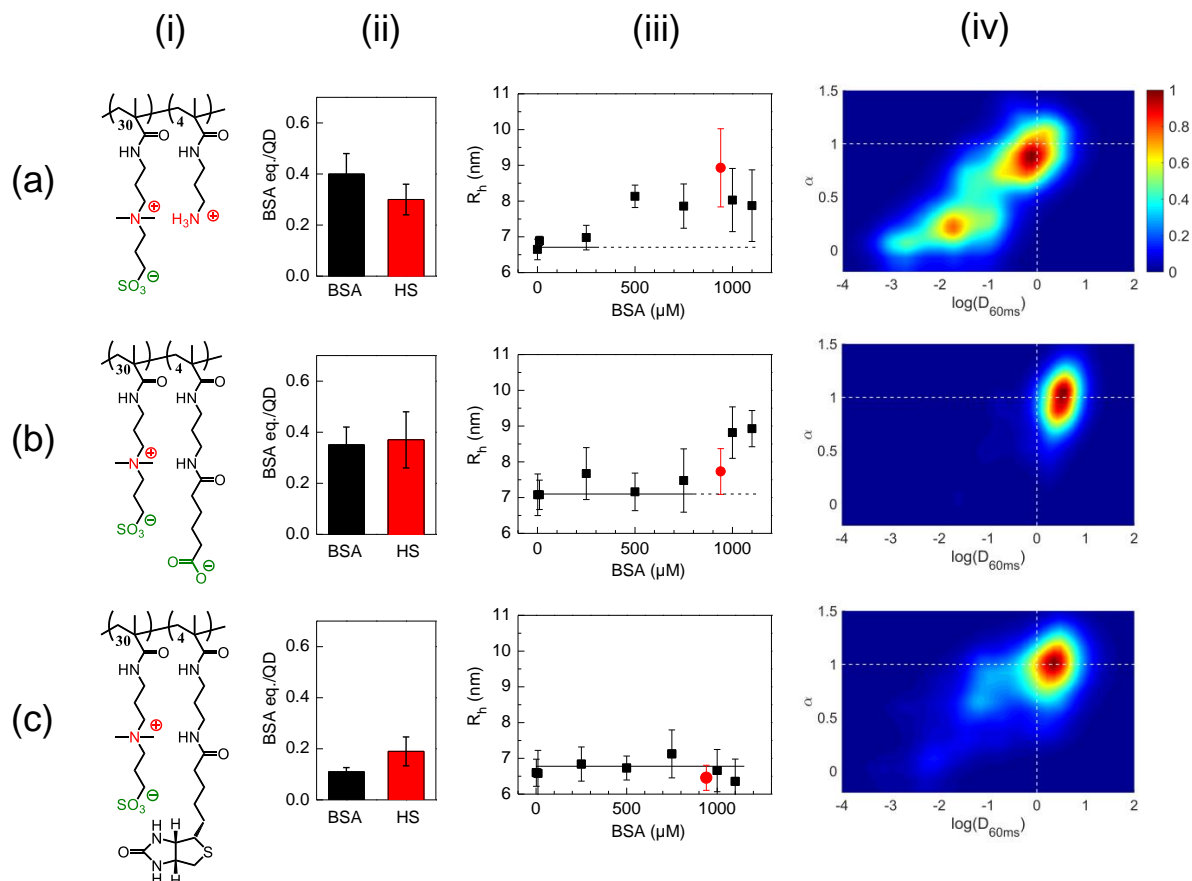


Figure 5. Influence of SB polymer modifications onto antifouling behavior. QDs are coated with (a) SB-NH₃⁺, (b) SB-COO⁻ and (c) SB-Biotin based ligands. (i) Structure of the modified SB, (ii) Average number of BSA equivalent adsorbed per QD within the hard corona, (iii) QD hydrodynamic radius determined by FCS and (iv) density plot in the α - $\log(D_{60\text{ms}})$ plane.

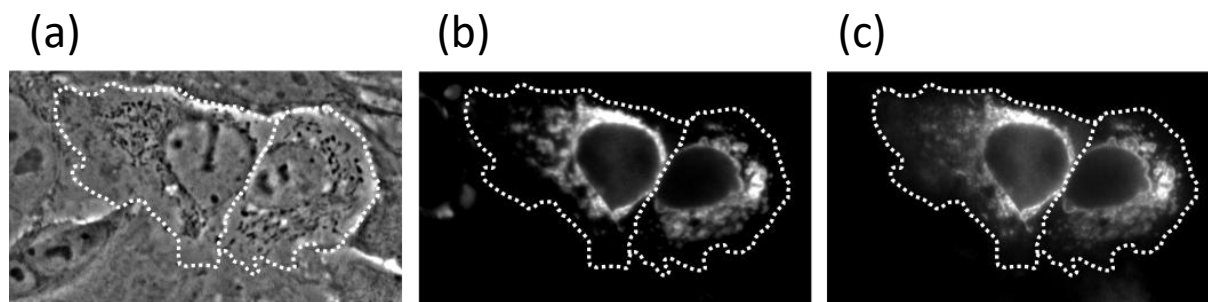


Figure 6. Targeting SB-Biotin QDs to an intracellular streptavidin-II-mRaspberry protein located in the endoplasmic reticulum of live HeLa cells. (a) Transmission, (b) mRaspberry fluorescence and (c) QD fluorescence images.

polyzwitterion polymer (Figures 2a and 5c). This suggests that in the case of extra amine or carboxylic functions, protein adsorption is mainly due to electrostatic interactions. This definitively confirms that the strict equality between the number of positive and negative charges - which is the intrinsic feature of zwitterions - is a necessary condition to limit protein-polymer interactions.

Fast Brownian diffusion is a prerequisite for imaging probes, so that they can freely explore the cytoplasmic environment and rapidly find their biomolecular target. Inertness is also key since nonspecific adsorption of biomolecules at the polyzwitterionic QD surface could also mask targeting moieties and prevent biomolecular recognition.[6] Furthermore, once attached to the target, they should not affect the dynamics or the localization of this target. To investigate whether functionalized SB-QDs remain fully functional in the intracellular environment, we microinjected SB-Biotin QDs in the cytoplasm of HeLa transfected with a plasmid expressing Ii, a protein located in the endoplasmic reticulum,[75] tagged with mRaspberry and streptavidin. The mRaspberry enables checking for effective transfection and localization of the fused protein target, while streptavidin provides a robust model target for biotin-SB QDs. Remarkably, in transfected cells, the vast majority of SB-Biotin QDs colocalized in less than a few seconds with their targets after microinjection (Figure 6). In contrast, SB-Biotin QDs microinjected in non-transfected cells filled the cytoplasm homogeneously (Figure S23) with a high diffusion coefficient (Figure 5c iv). This demonstrates that SB polyzwitterion nanoparticles are not only able to freely and rapidly diffuse in the cytoplasm, but also that small, neutral, targeting moieties remain easily accessible for biomolecular recognition.

CONCLUSION

With regards to the initial question, we have demonstrated that coating nanoparticles with zwitterionic polymers enable partial to

virtually complete elimination of both hard and soft protein corona. Interestingly, different zwitterions show distinct behavior in whole human serum, ranging from no detectable adsorption (sulfobetaine) to the formation of a monolayer-sized corona (phosphorylcholine) or partial nanoparticle aggregation (carboxybetaine). Modification of the charge balance by incorporation of a small fraction of charged functions into neutral polyzwitterions degrades antifouling properties, while functionalization with small neutral functions such as biotin preserves antifouling. Interestingly, we observed a strong correlation between in vitro behavior and cytoplasmic diffusion. In particular, polysulfobetaine coated nanoparticles demonstrate unhindered cytoplasmic Brownian motion. Biotin-modified SB QDs are able to bind efficiently to their intracellular targets. In the future, proteomic analysis of the composition of the protein corona at the surface of PC and CB coated nanoparticles would enable a deeper understanding of polyzwitterion-protein interactions. The exceptional antifouling properties of SB-coated nanoparticles open up exciting opportunities for designing nanoprobe for in vivo imaging or drug delivery. More generally, it is accepted that interactions between proteins and nanoparticles are responsible for capture by reticulo endothelial system and decrease the in vivo circulation time. The excellent stealth properties of sulfobetaine based ligands appear as a promising strategy to reduce immune recognition of nanoparticles.

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Data Availability.

The data required to reproduce these findings are available upon request.