Zwitterionic Silane Copolymer for Ultra-Stable and Bright Biomolecular Probes Based on Fluorescent Quantum Dot Nanoclusters

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Supporting Information

ABSTRACT: Fluorescent semiconductor quantum dots (QDs) exhibit several unique properties that make them suitable candidates for biomolecular sensing, including high brightness, photostability, broad excitation, and narrow emission spectra. Assembling these QDs into robust and functionalizable nanosized clusters (QD-NSCs) can provide fluorescent probes that are several orders of magnitude brighter than individual QDs, thus allowing an even greater sensitivity of detection with simplified instrumentation. However, the formation of compact, antifouling, functionalizable, and stable QD-NSCs remains a challenging task, especially for a use at ultralow concentrations for single-molecule detection. Here, we describe the development of fluorescent QD-NSCs envisioned as a tool for fast and sensitive biomolecular recognition. First, QDs were assembled into very compact 100−150 nm diameter spherical aggregates; the final QD-NSCs were obtained by growing a cross-linked silica shell around these aggregates. Hydrolytic stability in several concentration and pH conditions is a key requirement for a potential and efficient single-molecule detection tool. However, the hydrolysis of Si−O−Si bonds leads to desorption of monosilane-based surface groups at very low silica concentrations or in a slightly basic medium. Thus, we designed a novel multidentate copolymer composed of multiple silane as well as zwitterionic monomers. Coating silica beads with this multidentate copolymer provided a robust surface chemistry that was demonstrated to be stable against hydrolysis, even at low concentrations. Copolymer-coated silica beads also showed low fouling properties and high colloidal stability in saline solutions. Furthermore, incorporation of additional azido-monomers enabled easy functionalization of QD-NSCs using copper-free bio-orthogonal cyclooctyne-azide click chemistry, as demonstrated by a biotin−streptavidin affinity test.

KEYWORDS: zwitterionic copolymers, biodetection, quantum dots, nanoclusters, silica

1. INTRODUCTION

With the ever increasing demand for cheaper, faster and more sensitive bioassays, colloidal light-emitting semiconductor nanocrystals (quantum dots or QDs) have emerged as ideal candidates for biomolecular probe design. As compared to organic dyes, QDs are resistant to photobleaching and very bright: they can be used to achieve a highly sensitive detection.1−10 They also present narrow, composition- and size-dependent emission spectra, as well as a large absorption cross section that allows simultaneous excitation of several QD populations at a single wavelength; these unique properties have regularly been exploited for multiplexed detection.4−6

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Such inorganic particles are routinely synthesized in organic apolar solvents because this synthetic route enables a better control over their size, shape and composition.\textsuperscript{11} As a consequence, they are initially capped with hydrophobic ligands and their transfer into aqueous solutions for biological applications requires a modification of their surface chemistry. Several strategies have been developed to solubilize inorganic nanoparticles in water, including encapsulation in amphiphilic copolymers,\textsuperscript{2,7,12} growth of a silica shell,\textsuperscript{1,5,8,16–26} or ligand exchange, which consists in replacing the initial hydrophobic ligands with hydrophilic ones.\textsuperscript{9,11,26,27} The resulting water-soluble nanocrystals can be used in a wide variety of applications, including immunohistochemistry, FRET sensing, cell labeling and single protein tracking.\textsuperscript{1–10,15}

Recently, well-defined and colloidal stable spherical nanosized clusters (NSCs), obtained by assembling tens to thousands of individual nanoparticles, have attracted increasing interest.\textsuperscript{12,20,28} QD-NSCs, in particular, exhibit remarkable photostability and superior brightness that can be detected at the single particle level with higher efficiencies and simplified optical setups as compared to their individual QD counterparts, while also maintaining the unique QD spectral properties. QD-NSCs stand out as valuable tools for multiplexed detection of trace levels of biomarkers, down to the single molecule level without requiring signal amplification.\textsuperscript{2,5,8,9}

The simplest strategy to assemble hydrophobic nanoparticles such as QDs into hydrophobic and spherical colloidal nanosized aggregates is the well-established emulsification-evaporation technique.\textsuperscript{20,30,31} Briefly, emulsions of volatile organic solvent containing the nanoparticles are formed in water and stabilized by surfactants; the organic solvent is then evaporated, which yields dry assemblies of the initial nanoparticles. Notably, the choice of surfactant has a critical impact on the morphology and stability of the resulting clusters. It was demonstrated that protocols that involve small molecular surfactants lead to the formation of compact and size-controlled clusters.\textsuperscript{20,31} However, these small surfactants are labile and remain in a dynamic equilibrium of adsorption and desorption at the hydrophobic/hydrophilic interface. As a consequence, they tend to quickly desorb from the surface after dilution and washing steps, thus making them unsuitable for QD-NSCs functionalization. In contrast, longer amphiphilic copolymers remain strongly bound to the interface but reproducible well-controlled cluster shapes are difficult to obtain\textsuperscript{12,15} and hydrophobic polymer patches may remain exposed to the aqueous medium. Additionally, both types of coating may allow individual or small QD clusters to leak from the NSCs with time.\textsuperscript{23}

Considering all these reasons, we chose to grow a silica shell around each QD cluster. Not only does this cross-linked, gel-like shell prevent QD leakage from the QD-NSCs core, but it also enables an easier surface tailoring of NSCs.\textsuperscript{1,5,8,10,15,25,29} Surface functionalization of silica particles is well documented using alkoxysilanes, and this strategy works well in concentrated solutions. However, since the NSCs that we obtained are envisioned as single molecule detection probes, our surface chemistry should also be stable under highly dilute conditions. Silica particles are particularly affected by hydrolysis of Si–O–Si bonds at highly diluted concentrations. For example, Diedrich et al. reported hydrolysis rates of 10\textsuperscript{−7}–10\textsuperscript{−8} mol·g\textsuperscript{−1}·s\textsuperscript{−1} for silica beads at neutral pH.\textsuperscript{53} This, under infinite dilution conditions, corresponds to the loss of a monolayer equivalent on a 200 nm bead in only a few hours. Hydrolysis might be accelerated under various circumstances,\textsuperscript{34–36} e.g. in more basic medium, or in the presence of residual amino-silanes. While concentrated silica solutions remain stable due to favorable hydrolysis/condensation equilibrium, we demonstrate herein that the dissolution occurring in dilute conditions may lead to a rapid loss of surface silane moieties. To circumvent this limitation, we designed a novel copolymer composed of multiple anchoring silane groups to increase the stability of NSCs\textsuperscript{37} and zwiterionic motifs for aqueous solubility and low-fouling effect\textsuperscript{38–41} as well as reactive groups for further functionalization. When grafted on the surface of Stöber silica beads,\textsuperscript{42} the resulting polymer shows greater surface stability than monosilane ligands, provides excellent colloidal stability and limits nonspecific protein adsorption. Moreover, its functionalizable moiety enables efficient QD-NSCs bioconjugation using bio-orthogonal cyclooctyne-azide click chemistry.\textsuperscript{43} With these silica-embedded QD-NSC surface-modified by a novel copolymer, we finally demonstrate efficient biodetection by binding streptavidin to the particles, as a first model system.\textsuperscript{44}

2. MATERIALS AND METHODS

2.1. Chemicals. Acetic acid (CH\textsubscript{3}COOH, 99%, Aldrich), ammonium hydroxide (NH\textsubscript{4}OH, 28–30%, Aldrich), (3-aminopropyl)triethoxysilane (APTES, C\textsubscript{3}H\textsubscript{6}NO\textsubscript{Si}, ≥ 98%, Sigma-Aldrich), 2,2-azobis(2-amidinopropane) hydrochloride (V50, 97%, (=NC-(CH\textsubscript{3})\textsubscript{2}C(NH\textsubscript{2})\textsubscript{2}Cl) 2HCl, Aldrich), biotin-agarose (Sigma), bovine serum albumin (BSA, Sigma-Aldrich), cetyltrimethylammonium bromide (CTAB, C\textsubscript{19}H\textsubscript{42}BrN, 99%, Sigma-Aldrich), chloroform (CH\textsubscript{3}Cl\textsubscript{2}), deuterium oxide (D\textsubscript{2}O, 99.9 atom % D, Sigma-Aldrich), dihydrocyclooctyne-Cy3 dye (Cy3-DBCO, C\textsubscript{48}H\textsubscript{52}N\textsubscript{2}O\textsubscript{12}, Sigma-Aldrich), dibenzyclooctyne-N-hydroxysuccinimidyl ester (DBCOCO-NHS, C\textsubscript{21}H\textsubscript{15}N\textsubscript{2}O\textsubscript{2}, Aldrich), dimethyl sulfoxide (DMSO, CH\textsubscript{3}SO\textsubscript{2}, 99.8%, Sigma-Aldrich), ethanol absolute anhydrous (95%, C\textsubscript{2}H\textsubscript{5}OH, Carlo Erba), HEPES sodium salt (C\textsubscript{8}H\textsubscript{17}N\textsubscript{2}NaO\textsubscript{4}S, 99%, Sigma), hexane (C\textsubscript{6}H\textsubscript{14}), hydrochloric acid (HCl, Sigma-Aldrich), Igepal CO-520 (C\textsubscript{16}H\textsubscript{31}O\textsubscript{2}, n ~ 5, Sigma), 2-methacryloyloxyethyl phosphorylcholine (PC, C\textsubscript{37}H\textsubscript{55}NO\textsubscript{2}P, Sigma-Aldrich), methanol (95%, CH\textsubscript{3}OH, Carlo Erba), methanol-d\textsubscript{4} 99.8 atom % D (CD\textsubscript{2}OD, Sigma-Aldrich), rhodamine B isoiothiocyanate (RITC, C\textsubscript{43}H\textsubscript{64}Cl\textsubscript{10}N\textsubscript{10}S, Sigma-Aldrich), sodium acetate (C\textsubscript{2}H\textsubscript{4}NaO\textsubscript{2}, Sigma-Aldrich), sodium bicarbonate (NaHCO\textsubscript{3}, Sigma-Aldrich), sodium carbonate (Na\textsubscript{2}CO\textsubscript{3}, Prolabo), sodium chloride (NaCl, Sigma-Aldrich), sodium hydroxide (NaOH, 97%, Prolabo), sodium tetraborate (Na\textsubscript{2}B\textsubscript{4}O\textsubscript{7}, 99%, Sigma-Aldrich), streptavidin (STA, Biospa) and tetraethyorthosilicate, 98% (TEOS, C\textsubscript{4}H\textsubscript{9}O\textsubscript{4}Si, 99%, Sigma) were all used as-received.

2.2. Silica beads preparation and characterization. Silica beads were prepared according to a modified Stöber method\textsuperscript{42} described in Supporting Information. The product was precipitated multiple times in ethanol at 5000 g and resuspended in 10 mL ethanol.

Their size distribution was assessed by transmission electron microscopy (TEM) and dynamic light scattering (DLS). TEM images were taken with a JEOL 2010F. Samples were prepared by spreading a drop on an ultrathin 300 mesh Formvar/carbon-coated copper grid (Agar Scientific), which was degassed overnight. DLS measurements were carried out on a CGS-3 goniometer system equipped with a HeNe laser (633 nm) and an ALV/LSE-5003 correlator. All samples were initially transferred in a 10 mM HEPES, 150 mM NaCl, pH 7.4 buffer or 10 mM acetate, pH 4.5 buffer or 50 mM bicarbonate, pH 9.5 buffer by 3 rounds of centrifugation (6000 g, 5 min). Data was collected by monitoring the light intensity at different scattering angles between 30 and 150°. The hydrodynamic size distribution was obtained using the CONTIN algorithm as an intensity-averaged hydrodynamic radius.

The zeta potential of silica beads was determined with a ZetaSizer Nano ZS90 instrument (Malvern Instruments Ltd.). An estimated 0.1 m\textsuperscript{2} of silica beads was transferred into each of the 3 buffers used for DLS measurements and the samples were diluted about 1000 times.
Three series of 50 runs were performed in order to complete the measurements with the Zetasizer software.

2.3. Fluorescent QD-NSCs preparation. CdSe/CdS/ZnS QDs were synthesized as described in the Supporting Information. QD clusters were formed following an emulsion/evaporation protocol. First, 2 nmol of CdSe/CdS/ZnS QDs in hexane were precipitated with ethanol and centrifuged (14,000 g, 10 min). The supernatant was then removed and the QDs were redispersed in 200 μL of chloroform. 750 μL of a 4 nM CTAB aqueous solution was added under vigorous vortexing; the resulting mix was extruded by means of a 1 mL syringe into 0.80 μL of ethylene glycol. The resulting mix was then left to stir for 5 min and centrifuged (5000 g, 4 min). This procedure was repeated twice in order to enhance the surfactant exchange ratio on the surface of the clusters.

The clusters were then redispersed in 5 mL ethanol and coated with a silica shell according to a Stöber-modified method. After addition of 5 μL of triethoxysilane (TEOS) was added and the solution was stirred overnight at room temperature. The resulting QD-NSCs were precipitated multiple times in ethanol (600 g, 5 min) and finally resuspended in 5 mL of ethanol before being characterized by TEM. QD-NSCs were imaged by TEM as explained for the silica beads.

A photoluminescence spectrum of QD-NSCs was measured with an Edinburgh Instrument spectrometer using a 400 nm excitation wavelength. Fluorescence microscopy was used to compare the fluorescence signal of individual QD-NSCs to that of single QDs from the same synthesis batch. After a set of dilutions, images of individualized single QDs or QD-NSCs deposited onto a coverslip were acquired with a wide-field epifluorescence microscope (IX71 Olympus) using a 60× 1.2 NA (numerical aperture) water objective, a Chroma filter (λex= 450/25 and λem = 610 ± 20), and an electron-multiplying charge coupled device (EM CCD) camera (cascade 512B, Roper). The fluorescence signal of individual QD-NSCs relative to single QDs was evaluated using Image-J Software (NIH).

2.4. Synthesis and characterization of P(PC−PTMSi−N3) and P(PC−PTMSi−N3−APTES) copolymers. 2-methacryloyloxyethyl phosphorylcholine (PC, 1.5 g, 5 mmol, 1 equiv) and 3-(trimethoxysilyl)propyl methacrylate (PTMSi, 134 μL, 1 mmol, 0.2 equiv) monomers were mixed in methanol (30 mL) with 2,2-azobisis(2-aminopropane) hydrochloride (V50.9, 9.15 mg, 0.014 mmol, 0.015 equiv) initiator. The mixture was degassed by argon bubbling for 1 h at room temperature before being stirred overnight at 70 °C under argon atmosphere, resulting in a limpid colorless P(PC−PTMSi) solution.

The synthesis of P(PC−PTMSi−N3) was identical to the one described above, only with the addition of N-(11-azido-3,6,9-trioxadecan)-methacrylamide (−N3, 152 mg, 0.5 mmol, 0.1 equiv) to the initial PC, PTMSi mixture in methanol. The resulting P(PC−PTMSi−N3) solution was limpid and pale yellow. The protocol that describes the synthesis of N-(11-azido-3,6,9-trioxadecan)-methacrylamide is provided in the Supporting Information.

P(PC−PTMSi−N3) and P(PC−PTMSi−N3−APTES) were characterized by gel permeation chromatography (GPC, Viscotek GPC MAX, Viscotek VE 2001 GPC Solvent/Sample Module and TDA 302 triple detector array) in 0.5 M NaNO3 aqueous solution. 1H NMR spectroscopy was performed on a 400 MHz Bruker NMR spectrometer at 298 K. FT-IR spectra were performed on a Bruker vertex 70 equipped with an ATR. Purification of the polymer from remaining monomers was performed either using dialysis in methanol (3000 MW cutoff), drying under vacuum and resuspension in CD3OD or by 3 rounds of ultrafiltration (10 kDa, 10 min) with 3000 mL of vasoipin 500, 5600 MW cutoff 10 kDa). The polymer was further purified by dialysis in methanol (3000 MW cutoff) and freeze-drying. After being characterized by Dynamic Light Scattering (DLS) and their zeta potential was determined with a Malvern Zetasizer as described above for silica beads.

2.5. Polymer surface stability. The stability of the multidentate polymer P(PC−PTMSi−N3−APTES) derived organosilanes, was assessed on the silica beads’ surface for two different pH and concentration conditions. P(PC−PTMSi−Cy3)-coated beads were prepared as follows. For each condition, an estimated 0.5 m2 of P(PC−PTMSi−N3−APTES) was adsorbed on silica beads in methanol was reacted with dibenzocyclooctyne-Cy3 dye (Cy3-DBCO, 2 nmol). The subsequently obtained P(PC−PTMSi−Cy3)-coated silica beads were washed multiple times by centrifugation (6000 g, 5 min) in methanol before being resuspended in two different buffers at two different concentrations: (i) 10 mM HEPES, 150 mM NaCl, pH 7.4 buffer at a 0.025 m2·mL−1 concentration, (ii) 10 mM HEPES, 150 mM NaCl, pH 7.4 buffer at a 0.20 m2·mL−1 concentration and (iii) 0.1 M borate, pH 8 buffer at a 0.20 m2·mL−1 concentration.

The stability of these polymer-coated beads was compared to silica beads reacting with fluorescence-labeled APTES (f-APTES). F-APTES was obtained after mixing 0.5 nmol of fluorescein-N-hydroxysuccinimidy ester (fluorescein-NHS) prepared according to Giovanelli et al.9 with 0.5 mmol of APTES; the reaction was carried out overnight, in the dark and at room temperature. The product was used without further purification. For each condition, an estimated 0.5 m2 of silica beads in methanol was reacted with 5 μmol of F-APTES for 2 h at 60 °C. The subsequently obtained f-APTES-modified silica beads were washed multiple times by centrifugation (6000 g, 5 min) in methanol and resuspended in the same buffers and at the same concentrations as described for the P(PC−PTMSi−Cy3)-coated silica beads. P(PC−PTMSi−Cy3) and f-APTES-modified silica beads were stored at room temperature in the dark for 6 days. Polymer and monolamine grafting stability onto the silica surface was determined by following the absorbance of the pellet in search for evidence of surface-release polymer. Independent samples were considered at each time point.

For each absorbance measurement, DMSO was added to every sample in order to obtain a 2:1 DMSO: HEPES/NaCl buffer mixture for refractive index matching. After a centrifugation step (6000 g, 5 min) and sample resuspension in 500 μL, the absorbance at 494 nm (fluorescein) and at 570 nm (Cy3) was measured for the pellet with a UV−vis spectrometer (Shimadzu UV-1800).
2.6. Polymer-capped QD-NSCs. One mL of QD-NSCs were coated with P(PC−PTMSi) or P(PC−PTMSi-N$_3$) according to the same protocol as for the silica beads.

2.6.1. Bioconjugation on polymer-capped QD-NSCs. Dibenzocyclooctyne-functionalized streptavidin (STA-DBCO) was prepared by mixing a streptavidin solution (10 mg.mL$^{-1}$ in 0.1 M Borate buffer, pH 8) with DBCO-NHS (10 mg.mL$^{-1}$ in anhydrous DMSO) at a 1:3 molar ratio. In a typical reaction, 130 nmol of STA were mixed with 390 nmol of DBCO in ~800 μL of Borate buffer. The reaction was carried out for 1 h at room temperature and under agitation. Afterward, STA-DBCO was purified by three rounds of filtration (14000 g, 10 min) in vivaspin 500, MW cutoff 10 kDa (buffer = 10 mM HEPES, 150 mM NaCl, pH 7.4) and resuspended in 200 μL of the said buffer. One m$^2$ of P(PC−PTMSi-N$_3$)-coated QD-NSCs was subsequently reacted overnight by copper-free click-chemistry$^{43}$ with 100 μL of STA-DBCO in HEPES/NaCl. The two controls to this experiment consisted, on the one hand, in 1 m$^2$ of P(PC−PTMSi-N$_3$)-coated QD-NSCs, which was also mixed with STA-DBCO following the same procedure and, on the other hand, in 1 m$^2$ of P(PC−PTMSi-N$_3$)-coated QD-NSCs mixed with STA without a dibenzocyclooctyne moiety at the same molar ratio.

To demonstrate the effective binding of STA-DBCO to the azide-bearing P(PC−PTMSi-N$_3$)-coated QD-NSCs and its negligible binding to the negative control, the STA-modified sample and the controls were exposed to biotin-modified agarose beads. For that, 50 μL of commercial biotin agarose beads were washed in a 10 mM HEPES, 150 mM NaCl, pH 7.4 buffer and were left to react with the three batches of NSCs for 10 min at room temperature. After two washing rounds in the 7.4 buffer, images of the agarose beads deposited onto a coverslip were acquired with a wide-field epifluorescence microscope as described for QD-NSCs.

3. RESULTS AND DISCUSSION

3.1. Fluorescent QD-NSCs preparation. We first synthesized two types of particles. A modified Stöber protocol was used in order to synthesize silica beads with a mean diameter of 172 ± 1 nm as measured by TEM (Supporting Information, Figure S1).

The second type of particles was composed of CdSe/CdS/ZnS core/multishell QD clusters surrounded by an outer silica shell. As depicted in Scheme 1, QD clusters were formed by the emulsion-evaporation method from a QD solution in chloroform and an aqueous solution of CTAB. Compared to other usual surfactants such as the anionic SDS or nonionic Igepal, CTAB surfactants were found to provide clusters with compact spherical shapes and monomodal cluster size distribution.$^{30,31}$ Attempts to grow silica directly on these clusters resulted in rapid aggregation, which we believe is due to electrostatic interactions between negatively charged silica oligomers and positively charged clusters’ surfaces. CTAB was thus replaced by a nonionic PEGylated surfactant, Igepal CO-520, by means of a simple and rapid surfactant exchange. A Stöber-like protocol could then be used to grow a silica shell on the QD clusters, thanks to the colloidal stability of the PEG-coated clusters and the favorable interactions between the PEG layer and silica.

The conditions described in the experimental section provided rather monodisperse QD clusters, 87 ± 4 nm in diameter as analyzed by TEM (Figure 1). Smaller or larger average cluster sizes could be obtained by varying the amount of QDs in the organic phase. The thickness obtained for the silica layer was typically ~25 nm (Figure 1), which could be adjusted by changing the amount of TEOS, alcohol or ammonia.$^{17,22,42}$ To our knowledge, such monodisperse and very compact QD clusters entrapped in a silica layer have not yet been reported in literature.

Fluorescence spectroscopy of QD-NSCs showed that the assembly of QDs in clusters and coating with silica did not affect their fluorescence spectrum apart from a very small shift in the emission wavelength (Supporting Information, Figure S2). In order to validate the potential of these silica-coated QD clusters as bright fluorescence probes, the fluorescence signal of individual QD-NSCs was compared to that of single QDs from the same synthesis batch using fluorescence microscopy. Individual QD-NSCs were on average 1500 times brighter than single unmodified QDs. Considering that each QD-NSC presents a 90 nm-QD core and supposing that QDs are well-packed, an estimate of ~3700 QDs in each QD-NSCs can be drawn. This strongly suggests that QD-NSCs exhibit a relatively...
great fluorescence signal despite a limited quenching of the QDs following aggregation, transfer in water and silica growth. The modification of QD optical properties by silica coverage, in particular, was reported by Rogach et al. 38

3.2. Synthesis and characterization of P(PC−PTMSi) and P(PC−PTMSi-N3) copolymers. Bare silica is not adequate for biodetection applications due to strong non-specific adsorption. Silica surfaces are therefore usually modified by silane chemistry but these surfaces undergo hydrolysis over time, especially in extremely dilute conditions. We reasoned that including multiple anchoring silane groups in a single polymer chain would increase the stability of the organic surface coating compared to molecular monosilane groups. To this end, we synthesized a multidentate statistical copolymer, P(PC−PTMSi), composed of PTMSi to anchor the polymer on the silica surface and PC to enhance colloidal stability in saline solutions and to limit nonspecific adsorption of proteins thanks to the excellent antifouling effect of PC zwitterionic chains, in a 20:80 PTMSi-PC monomer ratio.40,41

Cyclooctyne-azide click chemistry is a popular and versatile bio-orthogonal conjugation scheme. In order to enable subsequent biomolecular coupling, we therefore chose to incorporate azido-terminated monomers in ~10% proportion in addition to the zwitterionic and silane monomers to produce the P(PC−PTMSi-N3) copolymer.

Both polymer syntheses yielded monomer conversion greater than 92%, as determined by 1H NMR. GPC analysis yielded number-average molecular weights (Mn) of 90 kDa with a polydispersity index of 2.5 for P(PC−PTMSi-N3). 1H NMR and IR spectra of purified polymers confirmed the presence of both silane and phosphorylcholine units (Supporting Information, Figures S6 and S7), even though FT-IR spectroscopy does not show the −N3 moiety. Assuming that the composition of the final polymer is similar to the composition of the initial monomer solution, we calculated an estimate of ~50 silanes per polymer chain; this should considerably increase the stability of the outer silica surface chemistry.

3.3. Polymer-capped silica beads preparation. Silica and QD-NSCs were coated with both copolymers by incubation in methanol at 60 °C for 2 h. Water traces were present in the reaction medium since the solvent was not anhydrous and water-mediated Stöber-like protocols were used to synthesize silica beads and QD-NSCs’ silica shells. This ensures efficient hydrolysis of the polymer silane groups on the surface of the silica beads. Following purification, TGA results indicated that P(PC−PTMSi) and P(PC−PTMSi-N3) effectively coat silica beads with a substantial coverage density of ~3 mg of polymer per m2 silica. The results for P(PC−PTMSi) are presented in Supporting Information (Supporting Information, Figure S8). No substantial differences in surface coverage were revealed for these two copolymers.

3.3.1. Colloidal stability and low-fouling properties. We compared the colloidal stability of P(PC−PTMSi)-coated silica beads with that of bare silica beads in acetate, HEPES/NaCl and bicarbonate buffer. Dynamic light scattering measurements (Supporting Information, Figure S9) showed that bare silica beads and P(PC−PTMSi)-coated silica beads exhibited a narrow-sized monomodal distribution around a 101 nm ±10 nm mean hydrodynamic radius value independently from the buffer. Measurements performed 1 week later on the same samples showed no change in the colloidal stability. Zwitterionic polymers are indeed known to provide high colloidal stability, even under extreme saline conditions. 9,42,43 Zeta potential measurements gave, for bare silica beads, an average value of −37.50 mV in acetate buffer pH 4.5, - 20.46 mV in HEPES/NaCl buffer pH 7.4 and −41.9 mV in bicarbonate buffer pH 9.5. The smaller potential in HEPES/NaCl can be explained by the charge screening in highly saline buffers. Once the silica beads are coated with P(PC−PTMSi), however, they exhibit much reduced zeta potentials, ranging from −11 mV to −5 mV independently from the buffer nature and pH. We attribute this low zeta potential to the neutral overall charge of phosphorylcholine, compared to the negatively charged silanol groups of bare silica. Residual negative zeta potentials may originate from different associated counterions and/or free, non surface-bound silane moieties. Altogether, these results confirm that we effectively succeeded in coating the silica beads with the polymer.

We then tested the antifouling properties of the copolymer coating by comparing protein adsorption on P(PC−PTMSi)-coated silica beads with that on bare silica beads, silica beads coated with a zwitterionic monosilane, the 3(dimethyl(3-(trimethoxysilyl)propyl)ammonio)propane-1-sulfonate (SBS). Dye-labeled BSA was used as a model protein. After a 30 min incubation of the beads in a concentrated RITC-labeled BSA solution (25 μM) and posterior removal of the unbound proteins by two rounds of centrifugation, the degree of nonspecific adsorption was probed by absorbance measurements at 555 nm (RITC). As shown in Figure 2, the peak absorbance value for bare silica beads is 8.5 times higher than that of P(PC−PTMSi)-coated silica beads. This means that there are 8.5 times more proteins adsorbed on the surface of bare silica than on the silica surface coated with the copolymer. This difference is due to the low fouling properties conferred by the zwitterionic phosphorylcholine moieties to P(PC−PTMSi)-coated silica beads and masking of the negatively charged silica surface.

The low-fouling properties of P(PC−PTMSi) are comparable to those of a sulfobetaine-functionalized monosilane. 35

3.3.2. Polymer surface stability. Silica surfaces are usually functionalized using monosilane groups such as those derived from aminopropyltriethoxysilane (APTES) or mercaptopropyltrimethoxysilane (MPTMS). 1,2,11,46,49 The covalent Si−O−Si
m².mL⁻¹), about 50% of dye-labeled APTES molecules were detached from the surface after 6 days of incubation. An even more significant and faster loss of APTES-bound dye molecules occurred under slightly basic conditions (pH 8) and led to a 60% loss in 6 days: we effectively observed an increase of hydrolysis with pH, as demonstrated by the pioneering works of Brady et al.35 Interestingly, incubating dye-APTES labeled silica beads in neutral pH conditions at lower concentration (0.025 m².mL⁻¹) led to a rapid loss of APTES-bound dye molecules, suggesting that dilute conditions favor displacement of the equilibrium toward hydrolysis. This loss of surface functionality represents a strong limitation, especially in the context of precise detection applications at the single molecule level, which occur by essence in extremely dilute conditions. In contrast, incubation of dye-labeled P(PC−PTMSi-N₃)-coated silica beads under the three conditions mentioned earlier show that the polymer remain strongly bound to the surface of silica beads after 6 days, even in slightly basic or dilute conditions. This increased stability can be attributed to the multidentate nature of the polymer, which presents many silane groups to bind to the nanoparticle surface.

3.4. Bioconjugation of the clusters. Cyclooctyne-azide click chemistry was chosen as a versatile bio-orthogonal conjugation technique. To demonstrate the potential of P(PC−PTMSi-N₃)-coated QD-NSCs for biodetection, we conjugated them to streptavidin and tested them on biotinylated substrates as a model targeting system. To this end, streptavidin was functionalized with DBCO, a strained alkyne moiety which enables cyclooctyne-azole coupling without the need for copper(I) catalyst. An NHS-ester − DBCO linker was randomly reacted with some lysines available on the streptavidin surface and the protein was purified by ultrafiltration. A 3:1 DBCO:NHS-streptavidin molar ratio was chosen to enable functionalization while keeping a low number of DBCO per streptavidin. Indeed, since we aim at creating a versatile platform, this procedure should be envisioned for reactions such as antibody conjugation and overconjugating antibodies can lead to their loss of functionality. P(PC−PTMSi-N₃)-coated QD-NSCs were then reacted with DBCO-streptavidin and purified by centrifugation without any appreciable loss of colloidal stability. Microscopic examination of biotinylated agarose beads incubated with these streptavidin-QD-NSCs showed a strong fluorescent signal (Figure 4(A)). By contrast, the interactions of the control, P(PC−PTMSi)−coated QD-NSCs, with biotin-bearing agarose surfaces appear as negligible, a fact that can be attributable to the low-fouling properties of the copolymer (Figure 4(B)). Altogether, these results demonstrate that DBCO-streptavidin binds to the P(PC−PTMSi-N₃)-coated QD-NSC specifically via cyclooctyne-azole conjugation and that the anchored streptavidin retains its biotin-recognition functionality.

An additional control consisting of QD-NSCs mixed with non-DBCO streptavidin also showed very little nonspecific fluorescent signal (Supporting Information, Figure S10). When non-DBCO streptavidin is mixed with the P(PC−PTMSi-N₃)-coated QD-NSCs, some residual nonspecific binding is observed, as shown above with BSA. Increasing the number of washing cycles after exposure to proteins (streptavidin or BSA in our case) could decrease nonspecific binding; an acceptable trade-off between controlling nonspecific binding and favoring a less laborious sample preparation process shall then be defined ad-hoc.

4. CONCLUSIONS

In this work, we presented a multistep strategy to design colloidal fluorescent nanosized clusters (NSCs) based on silica-embedded quantum dots (QDs). First, QD clusters were formed in a cetyltrimethylammonium bromide-mediated
microemulsion and a silica shell was subsequently grown on their surface by a Stöber-inspired process. This reproducible methodology led to compact and very bright clusters of approximately 130 nm diameter.

A particular focus was made on the surface chemistry tailoring of QD-NSCs with the synthesis and characterization of an original multidentate polymer-silane hybrid. After this polymer-silane hybrid was grafted on the surface of Stöber silica beads, we demonstrated that its zwitterionic chain was indeed responsible for a low-fouling effect with regards to a protein such as bovine serum albumine and for the colloidal stability of copolymer-capped silica beads in a physiological buffer. Moreover, the multiple silane anchoring functions of the polymer-silane hybrid seemed to contribute to its better anchoring on the surface of silica beads when compared to individual silane chains, which were more prone to unbinding over time due to hydrolysis.

After the polymer-silane hybrid was grafted onto the silica shell of the QD-NSCs, its azide moiety was effectively reacted with a cyclooctyne-functionalized protein as demonstrated by a specific biomolecular affinity test.

The nanobeads that have been designed constitute a versatile biosensing tool that can be tailored beyond the proof-of-concept showed in this paper e.g. by changing the nature of the inorganic particles encapsulated or the chemical functions available on the surface. Taken altogether, the results obtained provide interesting perspectives in terms of applications, for example as ultrasensitive single-molecule detection probes within complex biological samples or as signal amplifiers in rare target detection.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.7b01615.

- Synthesis and size characterization of bare silica beads,
- synthesis of QDs, TEM images, photoluminescence spectra, synthesis and characterization of the N-(11-azido-3,6,9-trioxaundecan)methacrylamide, additional characterization of P(PC−PTMSi-N₃), TGA results, intensity-weighted size distribution obtained from dynamic light scattering, and an additional control experiment to the bioconjugation experiment with STA without a dibenzocyclooctyne moiety. (PDF)

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**Notes**

The authors declare no competing financial interest.
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