

Article

Zwitterionic-silane copolymer for ultra stable and bright biomolecular probes based on fluorescent quantum dot nanoclusters.

Fatimata Dembele, Mariana Tasso, Laura Trapiella-Alfonso, Xiang Zhen Xu, Mohamed Hanafi, Nicolas Lequeux, and Thomas Pons

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20 *Fatimata DEMBELE¹, Mariana TASSO², Laura TRAPIELLA-ALFONSO^{1†}, Xiangzhen XU¹,*

21
22 *Mohamed HANAFI³, Nicolas LEQUEUX¹ and Thomas PONS^{1*}*

23
24
25
26 ¹ Laboratoire de Physique et d'Etude des Matériaux, ESPCI Paris, PSL Research University,
27
28 CNRS UMR8213, Université Pierre et Marie Curie, Sorbonne-Universités, 10 rue Vauquelin,
29
30 75005 Paris, France
31
32

33
34 ² Soft Matter Laboratory, INIFTA-CONICET, calle 64 y diagonal 113, 1906 La Plata, Argentina
35
36

37 ³ Laboratoire Sciences et Ingénierie de la Matière Molle, ESPCI Paris, PSL Research University,
38
39 CNRS UMR 7615, Université Pierre et Marie Curie, Sorbonne-Universités, 10 rue Vauquelin,
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41 75005 Paris, France
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48 **KEYWORDS.** Zwitterionic Copolymers, Biodetection, Quantum Dots, Nanoclusters, Silica
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3 **ABSTRACT.** Fluorescent semiconductor quantum dots (QDs) exhibit several unique properties
4 that make them suitable candidates for biomolecular sensing, including high brightness,
5 photostability, broad excitation and narrow emission spectra. Assembling these QDs into robust
6 and functionalizable nanosized clusters (QD-NSCs) can provide fluorescent probes that are
7 several orders of magnitude brighter than individual QDs, thus allowing an even greater
8 sensitivity of detection with simplified instrumentation. However, the formation of compact,
9 anti-fouling, functionalizable and stable QD-NSCs remains a challenging task, especially for a
10 use at ultra-low concentrations for single-molecule detection. Here, we describe the development
11 of fluorescent QD-NSCs envisioned as a tool for fast and sensitive biomolecular recognition.
12 Firstly, QDs were assembled into very compact 100-150 nm diameter spherical aggregates; the
13 final QD-NSCs were obtained by growing a cross-linked silica shell around these aggregates.
14 Hydrolytic stability in several concentration and pH conditions is a key requirement for a
15 potential and efficient single-molecule detection tool. However, hydrolysis of Si–O–Si bonds
16 leads to desorption of monosilane-based surface groups at very low silica concentrations or in a
17 slightly basic medium. Thus, we designed a novel multidentate copolymer, composed of multiple
18 silane as well as zwitterionic monomers. Coating silica beads with this multidentate copolymer
19 provided a robust surface chemistry that was demonstrated to be stable against hydrolysis, even
20 at low concentrations. Copolymer-coated silica beads also showed low-fouling properties and
21 high colloidal stability in saline solutions. Furthermore, incorporation of additional azido-
22 monomers enabled easy functionalization of QD-NSCs using copper-free bio-orthogonal
23 cyclooctyne-azide click chemistry, as demonstrated by a biotin-streptavidin affinity test.
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1. INTRODUCTION

With the ever increasing demand for cheaper, faster and more sensitive bioassays, colloidal light-emitting semi-conductor 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

Such inorganic particles are routinely synthesized in organic apolar solvents because this synthetic route enables a better control over their size, shape and composition.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,^{2,7,12-15} growth of a silica shell,^{1,5,8,16-26} or ligand exchange, which consists in replacing the initial hydrophobic ligands with hydrophilic ones.^{9,11,26,27} The resulting water-soluble nanocrystals can be used in a wide variety of applications, including immunohistochemistry, FRET sensing, cell labelling and single protein tracking.^{1-7,10,15}

Recently, well-defined and colloidally stable spherical nanosized clusters (NSCs), obtained by assembling tens to thousands of individual nanoparticles, have attracted increasing interest.^{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

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3 compared to their individual QD counterparts, while also maintaining the unique QD spectral
4 properties. QD-NSCs stand out as valuable tools for multiplexed detection of trace levels of
5 biomarkers, down the single molecule level without requiring signal amplification.^{2,3,8,29}
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10 The simplest strategy to assemble hydrophobic nanoparticles such as QDs into hydrophilic and
11 spherical colloidal nanosized aggregates is the well-established emulsification-evaporation
12 technique.^{20,30,31} Briefly, emulsions of volatile organic solvent containing the nanoparticles are
13 formed in water and stabilized by surfactants; the organic solvent is then evaporated, which
14 yields dry assemblies of the initial nanoparticles. Notably, the choice of surfactant has a critical
15 impact on the morphology and stability of the resulting clusters. It was demonstrated that
16 protocols that involve small molecular surfactants lead to the formation of compact and size-
17 controlled clusters.^{20,31} However, these small surfactants are labile and remain in a dynamic
18 equilibrium of adsorption and desorption at the hydrophobic/hydrophilic interface. As a
19 consequence, they tend to quickly desorb from the surface after dilution and washing steps, thus
20 making them unsuitable for QD-NSCs functionalization. In contrast, longer amphiphilic
21 copolymers remain strongly bound to the interface but reproducible well-controlled cluster
22 shapes are difficult to obtain^{2,12} and hydrophobic polymer patches may remain exposed to the
23 aqueous medium. Additionally, both types of coating may allow individual or small QDs clusters
24 to leak from the NSCs with time.²³
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44 Considering all these reasons, we chose to grow a silica shell around each QD cluster. Not only
45 does this cross-linked, gel-like shell prevent QD leakage from the QD-NSCs core, but it also
46 enables a rather easy surface tailoring of NSCs.^{1,5,8,16-23,25,32} Surface functionalization of silica
47 particles is well documented using alkoxy silanes, and this strategy works well in concentrated
48 solutions. However since the NSCs that we obtained are envisioned as single molecule detection
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3 probes, our surface chemistry should also be stable under highly dilute conditions. Silica
4 particles are particularly affected by hydrolysis of Si-O-Si bonds at highly-diluted
5 concentrations. For example, Diedrich et al. reported hydrolysis rates of 10^{-7} - 10^{-8} mol.g⁻¹. s⁻¹ for
6 silica beads at neutral pH.³³ This, under infinite dilution conditions, corresponds to the loss of a
7 monolayer equivalent on a 200 nm bead in only a few hours. Hydrolysis might be accelerated
8 under various circumstances,³⁴⁻³⁶ eg. in more basic medium, or in the presence of residual
9 amino-silanes. While concentrated silica solutions remain stable due to favorable
10 hydrolysis/condensation equilibrium, we demonstrate herein that the dissolution occurring in
11 dilute conditions may lead to a rapid loss of surface silane moieties. To circumvent this
12 limitation, we designed a novel copolymer composed of multiple anchoring silane groups to
13 increase the stability of NSCs,³⁷ zwitterionic motifs for aqueous solubility and low-fouling
14 effect,³⁸⁻⁴¹ as well as reactive groups for further functionalization. When grafted on the surface
15 of Stöber silica beads,⁴² the resulting polymer shows greater surface stability than monosilane
16 ligands, provides excellent colloidal stability and limits nonspecific protein adsorption.
17 Moreover, its functionalizable moiety enables efficient QD-NSCs bioconjugation using bio-
18 orthogonal cyclooctyne-azide click chemistry.⁴³ With these silica-embedded QD-NSC surface-
19 modified by a novel copolymer, we finally demonstrate efficient biodection by binding
20 streptavidin to the particles, as a first model system.⁴⁴

2. MATERIALS AND METHODS

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2.1. Chemicals. Acetic acid (CH₃COOH, 99%, Aldrich), ammonium hydroxide (NH₄OH, 28-30%, Aldrich), (3-aminopropyl) triethoxysilane (APTES, C₉H₂₃NO₃Si, ≥98%, Sigma-Aldrich),

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3 2,2-azobis(2-amidinopropane) hydrochloride (V50, 97%, $[=NC(CH_3)_2C(=NH)NH_2]_2 \cdot 2HCl$,
4 Aldrich), biotin-agarose (Sigma), bovine serum albumin (BSA, Sigma Aldrich),
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6 cetyltrimethylammonium bromide (CTAB, $C_{19}H_{42}BrN$, 99%, Sigma-Aldrich), chloroform
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8 ($CHCl_3$, VWR), deuterium oxide (D_2O , 99.9 atom % D, Sigma-Aldrich), dibenzocyclooctyne-
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10 Cy3 dye (Cy3-DBCO, $C_{62}H_{84}N_6O_{11}S_3$, Aldrich), dibenzocyclooctyne-N-hydroxysuccinimidyl
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12 ester (DBCO-NHS, $C_{23}H_{18}N_2O_5$, Aldrich), dimethyl sulfoxide (DMSO, C_2H_6SO , 99.8%, Sigma-
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14 Aldrich), ethanol absolute anhydrous (95%, C_2H_6O , Carlo Erba), HEPES sodium salt
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16 ($C_8H_{17}N_2NaO_4S$, 99%, Sigma), hexane (C_6H_{14} , VWR), hydrochloric acid (HCl, Sigma Aldrich),
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18 Igepal CO-520 ($(C_2H_4O)_n \cdot C_{15}H_{24}O$, $n \sim 5$, Sigma), 2-methacryloyloxyethyl phosphorylcholine
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20 (PC, $C_{11}H_{22}NO_6P$, Sigma-Aldrich), methanol (95%, CH_3OH , Carlo Erba), methanol-d4 99.8
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22 atom % D (CD_3OD , Sigma-Aldrich), rhodamine B isothiocyanate (RITC, $C_{29}H_{30}ClN_3O_3S$,
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24 Sigma-Aldrich), sodium acetate ($C_2H_3NaO_2$, Sigma-Aldrich), sodium bicarbonate ($NaHCO_3$,
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26 Sigma Aldrich), sodium carbonate (Na_2CO_3 , Prolabo), sodium chloride ($NaCl$, Sigma-Aldrich),
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28 sodium hydroxide ($NaOH$, 97%, Prolabo), sodium tetraborate ($Na_2B_4O_7$, 99%, Sigma-Aldrich),
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30 streptavidin (STA, Biospa) and tetraethyl orthosilicate, 98% (TEOS, $C_8H_{20}O_4Si$, 99%, Sigma)
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32 were all used as-received.
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42 **2.2. Silica beads preparation and characterization.** Silica beads were prepared according to
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44 a modified Stöber method⁴² described in Supporting Information. The product was precipitated
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46 multiple times in ethanol at 5000 g and resuspended in 10 mL ethanol.
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49 Their size distribution was assessed by transmission electron microscopy (TEM) and dynamic
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51 light scattering (DLS). TEM images were taken with a JEOL 2010F. Samples were prepared by
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53 spreading a drop on an ultra-thin 300 mesh Formvar/carbon-coated copper grid (Agar Scientific),
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3 which was degassed overnight. DLS measurements were carried out on a CGS-3 goniometer
4 system equipped with a HeNe laser (633 nm) and an ALV/LSE-5003 correlator. All samples
5 were initially transferred in a 10 mM HEPES, 150 mM NaCl, pH 7.4 buffer or 10 mM acetate,
6 pH 4.5 buffer or 50 mM bicarbonate, pH 9.5 buffer by 3 rounds of centrifugation (6000 g, 5
7 min). Data was collected by monitoring the light intensity at different scattering angles between
8 30 and 150°. The hydrodynamic size distribution was obtained using the CONTIN algorithm as
9 an intensity-averaged hydrodynamic radius.
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19 The zeta potential of silica beads was determined with a Zetasizer Nano ZS90 instrument
20 (Malvern Instruments Ltd.). An estimated 0.1 m² of silica beads was transferred into each of the
21 3 buffers used for DLS measurements and the samples were diluted about 1000 times. 3 series of
22 50 runs were performed in order to complete the measurements with the Zetasizer software.
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31 **2.3. Fluorescent QD-NSCs preparation.** CdSe/CdS/ZnS QDs were synthesized as described
32 in the Supporting Information. QD clusters were formed following an emulsion/evaporation
33 protocol. First, 2 nmol of CdSe/CdS/ZnS QDs in hexane were precipitated with ethanol and
34 centrifuged (14,000 g, 10 min). The supernatant was then removed and the QDs were redispersed
35 in 200 µL of chloroform. 750 µL of a 4 mM CTAB aqueous solution was added under vigorous
36 vortexing; the resulting mix was extruded by means of a 1 mL syringe through a 0.80x120mm
37 Sterican needle (Braun) until it became pale-orange and foamy. The mixture was then heated up
38 to 100°C for 10 minutes in order to evaporate the chloroform and to form stable QDs micelles.
39 Afterwards, 10 mL of Igepal CO-520 in pure water (200 mg.L⁻¹) were added to the suspension;
40 the mix was left to stir for 5 minutes and centrifuged (5000 g, 4 minutes). This procedure was
41 repeated twice in order to enhance the surfactant exchange ratio on the surface of the clusters.
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3 The clusters were then redispersed in 5 mL ethanol and coated with a silica shell according to a
4 Stöber-modified method.⁴² After addition of pure water (1.5 mL), the pH of the suspension was
5 raised with 160 μ L of a 28% NH_4OH solution. Finally, 50 μ L of triethoxysilane (TEOS) was
6 added and the solution was stirred overnight at room temperature. The resulting QD-NSCs were
7 precipitated multiple times in ethanol (6000 g, 5 min) and finally resuspended in 5 mL of ethanol
8 before being characterized by TEM. QD-NSCs were imaged by TEM as explained for the silica
9 beads.
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19 A photoluminescence spectrum of QD-NSCs was measured with an Edinburgh Instrument
20 spectrometer using a 400 nm excitation wavelength. Fluorescence microscopy was used to
21 compare the fluorescence signal of individual QD-NSCs to that of single QDs from the same
22 synthesis batch. After a set of dilutions, images of individualized single QDs or QD-NSCs
23 deposited onto a coverslip were acquired with a wide-field epifluorescence microscope (IX71
24 Olympus) using a 60×1.2 NA (numerical aperture) water objective, a Chroma filter ($\lambda_{\text{exc}} =$
25 $450/25$ and $\lambda_{\text{em}} = 610 \pm 20$), and an electron-multiplying charge coupled device (EM CCD) camera
26 (cascade 512B Roper). The fluorescence signal of individual QD-NSCs relative to single QDs
27 was evaluated using Image-J Software (NIH).
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42 **2.4. Synthesis and characterization of P(PC-PTMSi) and P(PC-PTMSi-N₃) copolymers.**

43 2-methacryloyloxyethyl phosphorylcholine (PC, 1.5 g, 5 mmol, 1 equiv.) and 3-
44 (trimethoxysilyl)propyl methacrylate (PTMSi, 134 μ L, 1 mmol, 0.2 equiv.) monomers were
45 mixed in methanol (30 mL) with 2,2-azobis(2-amidinopropane) hydrochloride (V50, 9.15 mg,
46 0.014 mmol, 0.015 equiv.) initiator. The mixture was degassed by argon bubbling for 1 hour at
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3 room temperature before being stirred overnight at 70°C under argon atmosphere, resulting in a
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5 limpid colorless P(PC-PTMSi) solution.
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8 The synthesis of P(PC-PTMSi-N₃) was identical to the one described above, only with the
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10 addition of *N*-(11-azido-3,6,9-trioxaundecan)methacrylamide (-N₃, 152 mg, 0.5 mmol, 0.1
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12 equiv.) to the initial PC, PTMSi mixture in methanol. The resulting P(PC-PTMSi-N₃) solution
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14 was limpid and pale yellow. The protocol that describes the synthesis of *N*-(11-azido-3,6,9-
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16 trioxaundecan)methacrylamide is provided in Supporting Information.
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20 P(PC-PTMSi) and P(PC-PTMSi-N₃) were characterized by gel permeation chromatography
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22 (GPC, Viscotek GPC MAX, Viscotek VE 2001 GPC Solvent/Sample Module and TDA 302
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24 triple detector array) in 0.5 M NaNO₃ aqueous solution. ¹H NMR spectroscopy was performed
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26 on a 400 MHz Bruker NMR spectrometer at 298 K. FT-IR spectra were performed on a Bruker
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28 vertex 70 equipped with an ATR. Purification of the polymer from remaining monomers was
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30 performed either using dialysis in methanol (3000 MW cutoff), drying under vacuum and
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32 resuspension in CD₃OD or by 3 rounds of ultrafiltration (14000 g, 10 min) with D₂O in vivaspin
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34 500, MW cutoff 10 kDa).
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40 **2.5. Polymer-capped silica beads preparation.** An estimated of 1 m² surface equivalent silica
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42 beads were transferred into 100 μL methanol and reacted for 2 hours at 60°C with 400 μL of
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44 P(PC-PTMSi) or P(PC-PTMSi-N₃) solutions in methanol, corresponding to about 40 mg
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46 polymer. The products, P(PC-PTMSi)- and P(PC-PTMSi-N₃)-coated silica beads, were
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48 precipitated multiple times in methanol. P(PC-PTMSi)-coated silica beads in methanol were
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50 characterized by thermogravimetric analysis (TGA, SDT Q-600 modulus, TA Instruments).
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3 Their colloidal stability was assessed by Dynamic Light Scattering (DLS) and their zeta potential
4 was determined with a Malvern Zetasizer as described above for silica beads.
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8 **2.5.1 Low-fouling properties.** The nonspecific adsorption of proteins was tested on P(PC-
9 PTMSi)-coated silica beads as compared to bare silica beads and silica beads coated with a
10 model zwitterionic monosilane, the 3(dimethyl-(3-(trimethoxysilyl)propyl)ammonio)propane-1-
11 sulfonate (SBS). SBS and SBS-coated silica beads were prepared according to the protocol
12 described by Estephan et al.⁴⁵
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19 The test was conducted as follows: rhodamine isothiocyanate (RITC)-labeled bovine serum
20 albumin (BSA) was prepared by adding 40 μL of 5 $\text{mg}\cdot\text{mL}^{-1}$ RITC in DMSO to 400 μL of a 0.1
21 M Borate, pH 8 buffer solution containing 4 mg BSA. After being reacted with the dye at 4°C
22 overnight, the labeled proteins were purified by NAP-10 exclusion chromatography (GE
23 Healthcare Life Sciences) and two rounds of ultrafiltration (14000 g, 10 min) in vivaspin 500,
24 MW cutoff 30 kDa). The BSA-RITC proteins were finally resuspended in 10 mM HEPES, 150
25 mM NaCl, pH 7 buffer and their concentration was estimated using absorption measurements at
26 280 nm. An estimated 0.1 m^2 bare silica, SBS-coated silica beads or P(PC-PTMSi)-coated silica
27 beads were diluted in 100 μL of HEPES/NaCl buffer, mixed with 100 μL BSA-RITC (25 μM)
28 and agitated for 30 minutes at room temperature. The beads were then purified by 3 cycles of
29 centrifugation at 10,000 g for 10 minutes and resuspension in HEPES/NaCl buffer. The beads
30 were finally resuspended in a 2:1 DMSO: HEPES/NaCl buffer mixture for refractive index
31 matching and their absorbance at 555 nm was measured to quantify the amount of adsorbed
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3 **2.5.2 Polymer surface stability.** The stability of the multidentate polymer P(PC-PTMSi-N₃)
4 versus APTES-derived organosilanes, was assessed on the silica beads' surface for two different
5 pH and concentration conditions.
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10 P(PC-PTMSi-Cy3)-coated beads were prepared as follows. For each condition, an estimated
11 0.5 m² of P(PC-PTMSi-N₃)-coated silica beads in methanol was reacted with
12 dibenzocyclooctyne-Cy3 dye (Cy3-DBCO, 2 nmol). The subsequently obtained P(PC-PTMSi-
13 Cy3)-coated silica beads were washed multiple times by centrifugation (6000 g, 5 min) in
14 methanol before being resuspended in two different buffers and at two different concentrations:
15 (i) 10 mM HEPES, 150 mM NaCl, pH 7.4 buffer at a 0.025 m².mL⁻¹ concentration, (ii) 10 mM
16 HEPES, 150 mM NaCl, pH 7.4 buffer at a 0.20 m².mL⁻¹ concentration and (iii) 0.1 M borate, pH
17 8 buffer at a 0.20 m².mL⁻¹ concentration.
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21 The stability of these polymer-coated beads was compared to silica beads reacted with
22 fluorescein-labeled APTES (f-APTES). f-APTES was obtained after mixing 0.5 mmol of
23 fluorescein-N-hydroxysuccinimidyl ester (fluorescein-NHS) prepared according to Giovanelli et
24 al.⁹ with 0.5 mmol of APTES; the reaction was carried out overnight, in the dark and at room
25 temperature. The product was used without further purification. For each condition, an estimated
26 0.5 m² of silica beads in methanol was reacted with 5 μmol of f-APTES for 2 hours at 60°C. The
27 subsequently obtained f-APTES-modified silica beads were washed multiple times by
28 centrifugation (6000 g, 5 min) in methanol and resuspended in the same buffers and at the same
29 concentrations as described for the P(PC-PTMSi-Cy3)-coated silica beads.
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33 P(PC-PTMSi-Cy3) and f-APTES-modified silica beads were stored at room temperature in the
34 dark for 6 days. Polymer and monosilane grafting stability onto the silica surface was determined
35 by following the absorbance of the pellet in search for evidence of surface-released polymer.
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3 Independent samples were considered at each time point. For each absorbance measurement,
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5 DMSO was added to each sample in order to obtain a 2:1 DMSO: HEPES/NaCl buffer mixture
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7 for refractive index matching. After a centrifugation step (6000 g, 5 min) and sample
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9 resuspension in 500 μL , the absorbance at 494 nm (fluorescein) and at 570 nm (Cy3) was
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11 measured for the pellet with a UV-vis spectrometer (Shimadzu UV-1800).
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17 **2.6. Polymer-capped QD-NSCs.** 1 mL of QD-NSCs were coated with P(PC-PTMSi) or P(PC-
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19 PTMSi-N₃) according to the same protocol as for the silica beads.
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21 **2.6.1 Bioconjugation on polymer-capped QD-NSCs.** Dibenzocyclooctyne-functionalized
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23 streptavidin (STA-DBCO) was prepared by mixing a streptavidin solution (10 mg.mL⁻¹ in 0.1 M
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25 Borate buffer, pH 8) with DBCO-NHS (10 mg.mL⁻¹ in anhydrous DMSO) at a 1:3 molar ratio. In
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27 a typical reaction, 130 nmol of STA were mixed with 390 nmol of DBCO in ~ 800 μL of Borate
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29 buffer. The reaction was carried out for 1 hour at room temperature and under agitation.
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31 Afterwards, STA-DBCO was purified by three rounds of filtration (14000 g, 10 min) in vivaspin
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33 500, MW cutoff 10 kDa (buffer = 10 mM HEPES, 150 mM NaCl, pH 7.4) and resuspended in
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35 200 μL of the said buffer. 1 m² of P(PC-PTMSi-N₃)-coated QD-NSCs was subsequently reacted
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37 overnight by copper-free click-chemistry⁴³ with 100 μL of STA-DBCO in HEPES/NaCl . The
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39 two controls to this experiment consisted, on the one hand, in 1 m² of P(PC-PTMSi)-coated QD-
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41 NSCs, which was also mixed with STA-DBCO following the same procedure and, on the other
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43 hand, in 1 m² of P(PC-PTMSi-N₃)-coated QD-NSCs mixed with STA without a
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45 dibenzocyclooctyne moiety at the same molar ratio.
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51 To demonstrate the effective binding of STA-DBCO to the azide-bearing P(PC-PTMSi-N₃)-
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53 coated QD-NSCs and its negligible binding to the negative control, the STA-modified sample
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3 and the controls were exposed to biotin-modified agarose beads. For that, 50 μ L of commercial
4 biotin agarose beads were washed in a 10 mM HEPES, 150 mM NaCl, pH 7.4 buffer and were
5 left to react with the three batches of NSCs for 10 min at room temperature. After two washing
6 rounds in the 7.4 buffer, images of the agarose beads deposited onto a coverslip were acquired
7 with a wide-field epifluorescence microscope as described for QD-NSCs.
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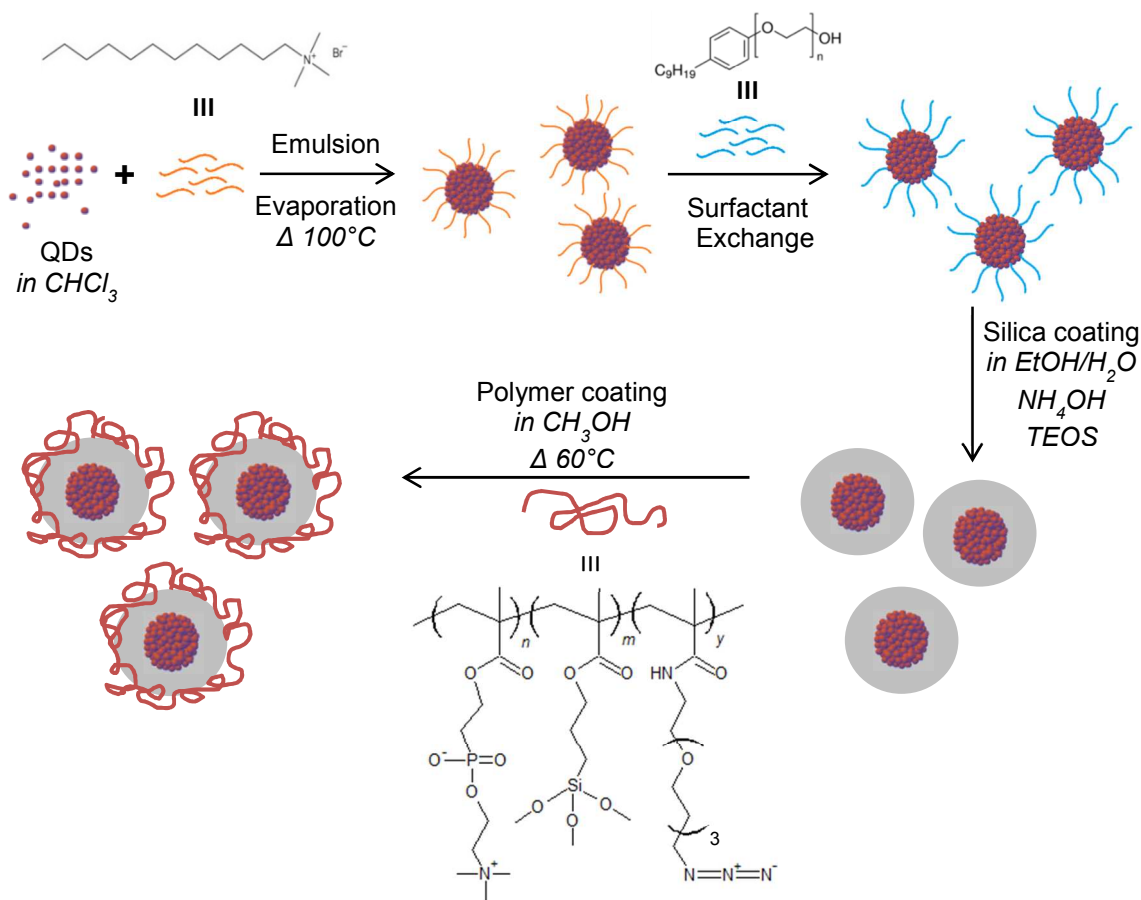
18 **3. RESULTS AND DISCUSSION**

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25 **3.1. Fluorescent QD-NSCs preparation.** We first synthesized two types of particles. A
26 modified Stöber protocol was used in order to synthesize silica beads with a mean diameter of
27 172 \pm 1 nm as measured by TEM (Supporting Information, Figure S1).
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32 The second type of particles was composed of CdSe/CdS/ZnS core/multishell QD clusters
33 surrounded by an outer silica shell. As depicted in Scheme 1, QD clusters were formed by the
34 emulsion-evaporation method from a QD solution in chloroform and an aqueous solution of
35 CTAB. Compared to other usual surfactants such as the anionic SDS or nonionic Igepal, CTAB
36 surfactants were found to provide clusters with compact spherical shapes and monomodal cluster
37 size distribution.^{20,31} Attempts to grow silica directly on these clusters resulted in rapid
38 aggregation, which we believe is due to electrostatic interactions between negatively charged
39 silica oligomers and positively charged clusters' surfaces. CTAB was thus replaced by a nonionic
40 PEGylated surfactant, Igepal CO-520, by a simple and rapid dynamic surfactant exchange. A
41 Stöber-like protocol could then be used to grow a silica shell on the QD clusters, thanks to the
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colloidal stability of the PEG-coated clusters and the favorable interactions between the PEG layer and silica.

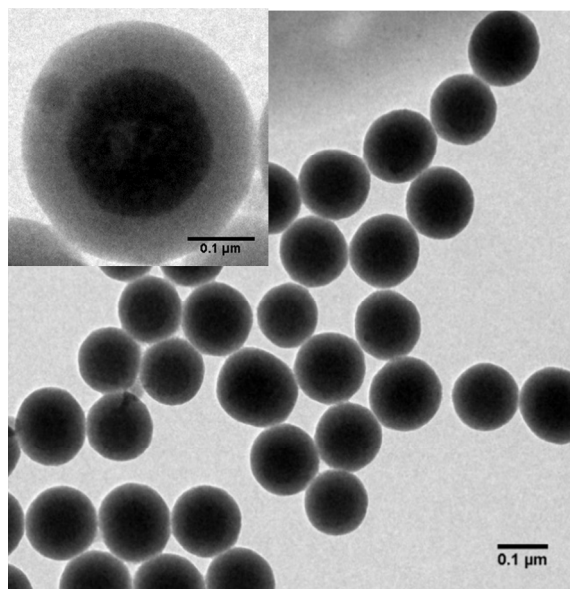
Scheme 1. Preparation of QD-NSC coated with a silica shell and P(PC-PTMSi-N₃) copolymer.



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

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3 monodisperse and very compact QD clusters entrapped in a silica layer have not yet been
4 reported in literature.
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8 Fluorescence spectroscopy of QD-NSCs showed that the assembly of QDs in clusters and
9 coating with silica did not affect their fluorescence spectrum apart from a very small shift in the
10 emission wavelength (Supporting Information, Figure S2). In order to validate the potential of
11 these silica-coated QD clusters as bright fluorescence probes, the fluorescence signal of
12 individual QD-NSCs was compared to that of single QDs from the same synthesis batch using
13 fluorescence microscopy. Individual QD-NSCs were on average 1500 times brighter than single
14 unmodified QDs. Considering that each QD-NSC presents a 90 nm-QD core and supposing that
15 QDs are well-packed, an estimate of ~ 3700 QDs in each QD-NSCs can be drawn. This strongly
16 suggests that QD-NSCs exhibit a relatively great fluorescence signal despite a limited quenching
17 of the QDs following aggregation, transfer in water and silica growth. The modification of QD
18 optical properties by silica coverage, in particular, was reported by Rogach et al..²⁸
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6 **Figure 1:** TEM image of silica-coated QD clusters (QD-NSCs). The average size of the clusters
7 presented in this work is of 137 ± 8 nm, which makes them atypically compact as well as
8 monodisperse. Inset: higher magnification of a different sample obtained by slightly increasing
9 the amount of QDs, which resulted in bigger QD-NSCs.
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18 **3.2. Synthesis and characterization of P(PC-PTMSi) and P(PC-PTMSi-N₃) copolymers.**

19 Bare silica is not adequate for biodetection applications due to strong nonspecific adsorption.
20 Silica surfaces are therefore usually modified by silane chemistry but these surfaces undergo
21 hydrolysis over time, especially in extremely dilute conditions. We reasoned that including
22 multiple anchoring silane groups in a single polymer chain would increase the stability of the
23 organic surface coating compared to molecular mono-silane groups. To this end, we synthesized
24 a multidentate statistical copolymer, P(PC-PTMSi), composed of PTMSi to anchor the polymer
25 on the silica surface and PC to enhance colloidal stability in saline solutions, limit nonspecific
26 adsorption of proteins thanks to the excellent antifouling effect of PC zwitterionic chains, in a
27 20:80 PTMSi-PC monomer ratio^{40,41}.
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40 Cyclooctyne-azide click chemistry is a popular and versatile bio-orthogonal conjugation
41 scheme. In order to enable subsequent biomolecular coupling, we therefore chose to incorporate
42 azido-terminated monomers in ~10 % proportion in addition to the zwitterionic and silane
43 monomers to produce the P(PC-PTMSi-N₃) copolymer.
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50 Both polymer syntheses yielded monomer conversion greater than 92%, as determined by ¹H-
51 NMR. GPC analysis yielded -average molecular weights (M_n) of 90 kDa with a polydispersity
52 index of 2.5 for P(PC-PTMSi-N₃). ¹H NMR and IR spectra of purified polymers confirmed the
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3 presence of both silane and phosphorylcholine units (Supporting Information, Figures S6 and
4 S7), even though FT-IR spectroscopy doesn't show the $-N_3$ moiety. Assuming that the
5 composition of the final polymer is similar to the composition of the initial monomer solution,
6 we calculated an estimate of ~ 50 silanes per polymer chain; this should considerably increase
7 the stability of the outer silica surface chemistry.
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17 **3.3. Polymer-capped silica beads preparation.** Silica and QD-NSCs were coated with both
18 copolymers by incubation in methanol at 60°C for 2 hours. Water traces were present in the
19 reaction medium since the solvent was not anhydrous and water-mediated Stöber-like protocols
20 were used to synthesize silica beads and QD-NSCs' silica shells. This ensures efficient
21 condensation of the polymer silane groups on the surface of the silica beads^{36,46,47}. Following
22 purification, TGA results indicated that P(PC-PTMSi) and P(PC-PTMSi- N_3) effectively coat
23 silica beads with a substantial coverage density of ~ 3 mg of polymer per m^2 silica. The results
24 for P(PC-PTMSi) are presented in Supporting Information (Supporting Information, Figure S8).
25 No substantial differences in surface coverage were revealed for these two copolymers.
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38 **3.3.1 Colloidal stability and low-fouling properties.** We compared the colloidal stability of
39 P(PC-PTMSi)-coated silica beads with that of bare silica beads in acetate, HEPES/NaCl and
40 bicarbonate buffer. Dynamic light scattering measurements (Supporting Information, Figure S9)
41 showed that bare silica beads and P(PC-PTMSi)-coated silica beads exhibited a narrow-sized
42 monomodal distribution around a 101 nm \pm 10 nm mean hydrodynamic radius value
43 independently from the buffer. Measurements performed one week later on the same samples
44 showed no change in the colloidal stability. Zwitterionic polymers are indeed known to provide
45 high colloidal stability, even under extreme saline conditions.^{9,47,48} Zeta potential measurements
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3 gave, for bare silica beads, an average value of -37.50 mV in acetate buffer pH 4.5, -20.46 mV
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5 in HEPES/NaCl buffer pH 7.4 and -41.9 mV in bicarbonate buffer pH 9.5. The smaller potential
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7 in HEPES/NaCl can be explained by the charge screening in highly saline buffers. Once the
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9 silica beads are coated with P(PC-PTMSi), however, they exhibit much reduced zeta potentials,
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11 ranging from -11 mV to -5 mV independently from the buffer nature and pH. We attribute this
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13 low zeta potential to the neutral overall charge of phosphorylcholine, compared to the negatively
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15 charged silanol groups of bare silica. Residual negative zeta potentials may originate from
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17 different associated counterions and/or free, non surface-bound silane moieties. Altogether, these
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19 results confirm that we effectively succeeded in coating the silica beads with the polymer.
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24 We then tested the antifouling properties of the copolymer coating by comparing protein
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26 adsorption on P(PC-PTMSi)-coated silica beads with that on bare silica beads, silica beads
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28 coated with a zwitterionic monosilane, the 3(dimethyl-(3-
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30 (trimethoxysilyl)propyl)ammonio)propane-1-sulfonate (SBS). Dye-labeled BSA was used as a
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32 model protein. After a 30 min incubation of the beads in a concentrated RITC-labeled BSA
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34 solution ($25 \mu\text{M}$) and posterior removal of the unbound proteins by two rounds of centrifugation,
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36 the degree of nonspecific adsorption was probed by absorbance measurements at 555 nm
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38 (RITC). As shown in Figure 2, the peak absorbance value for bare silica beads is 8.5 times higher
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40 than that of P(PC-PTMSi)-coated silica beads. This means that there are 8.5 times more proteins
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42 adsorbed on the surface of bare silica than on the silica surface coated with the copolymer. This
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44 difference is due to the low fouling properties conferred by the zwitterionic phosphorylcholine
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46 moieties to P(PC-PTMSi)-coated silica beads and masking of the negatively charged silica
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The low-fouling properties of P(PC-PTMSi) are comparable to those of a sulfobetaine-functionalized monosilane⁴⁵.

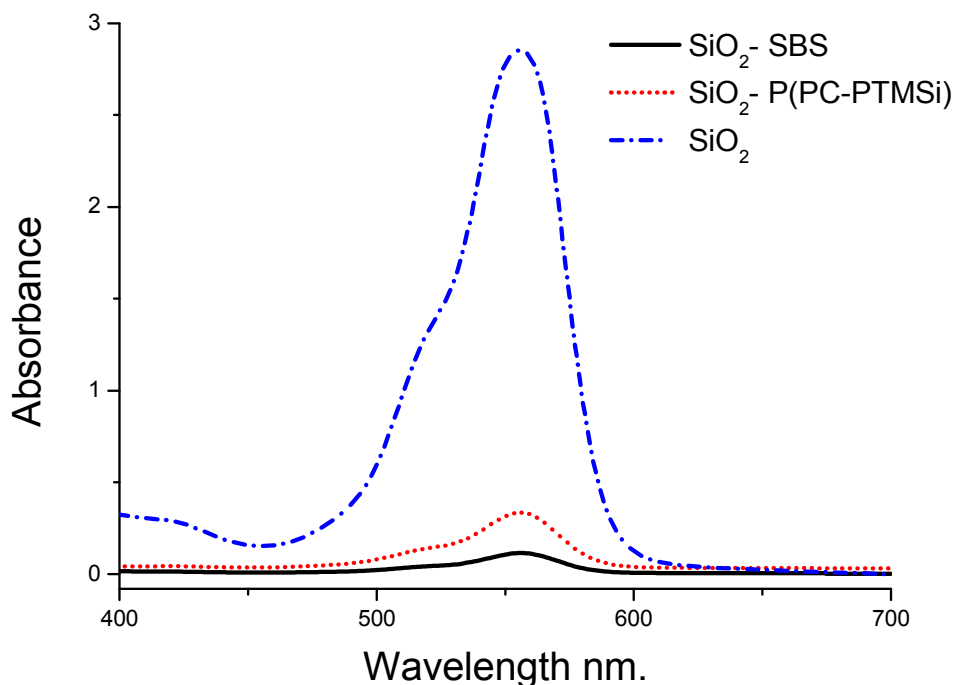


Figure 2: Absorbance values measured for bare silica (SiO_2), P(PC-PTMSi)-coated silica beads (SiO_2 - P(PC-PTMSi)) and SBS-coated silica beads (SiO_2 - SBS) exposed to RITC-labeled BSA and washed. The characteristic absorbance maximum of RITC, corresponding to the RITC-labeled BSA, can be found at 555nm.

3.3.2 Polymer surface stability. Silica surfaces are usually functionalized using monosilane groups such as those derived from aminopropyltriethoxysilane (APTES) or mercaptopropyltrimethoxysilane (MPTMS)^{1,21,46,49}. The covalent Si-O-Si bonds between these molecules and silanol groups on the silica surface are however in constant condensation/hydrolysis equilibrium in aqueous media. Hydrolysis rates can be increased by

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3 varying many parameters like temperature, salinity, particle size or pH^{33–36,50}: therefore, its
4 effects can be critical to preserve the beads' low fouling properties and functional moieties.
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8 In this work, we presumed that establishing multiple siloxane bonds would enhance the
9 stability of the polymer on the silica surface. In order to test this hypothesis, the hydrolytic
10 stability of our multidentate polymer was compared to monosilane molecules under three
11 conditions: diluted at physiological pH ($0.025 \text{ m}^2 \cdot \text{mL}^{-1}$ at pH 7.4), concentrated at physiological
12 pH ($0.2 \text{ m}^2 \cdot \text{mL}^{-1}$ at pH 7.4) and concentrated at slightly basic pH ($0.2 \text{ m}^2 \cdot \text{mL}^{-1}$ at pH 8). First, we
13 functionalized silica beads with P(PC-PTMSi-N₃) and labeled them with a Cy3 dye using
14 cyclooctyne-based copper-free click chemistry. Control silica beads coated with a P(PC-PTMSi)
15 copolymer without azido groups showed no labeling. Then, we modified APTES with
16 fluorescein-NHS ester and thereafter proceeded to bind the resulting f-APTES molecule to silica
17 beads. Both, fluorescently-labeled APTES and multidentate polymer samples were left to
18 incubate, in the dark and at room temperature, in the three different conditions mentioned above.
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20 At each evaluated time point, a small sample of $\sim 0.1 \text{ m}^2$ was taken and centrifuged once to
21 separate free dye-labeled molecules from those bound to the silica beads. Absorbance
22 measurements of resuspended pellets enabled us to estimate the fraction of functionalization
23 molecules still bound to the beads.
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27 The results are displayed on Figure 3. In a physiological pH buffer and in a relatively
28 concentrated solution of beads ($0.2 \text{ m}^2 \cdot \text{mL}^{-1}$), about 50% of dye-labeled APTES molecules were
29 detached from the surface after 6 days of incubation. An even more significant and faster loss of
30 APTES-bound dye molecules occurred under slightly basic conditions (pH 8) and led to a 60%
31 loss in 6 days: we effectively observed an increase of hydrolysis with pH, as demonstrated by the
32 pioneering works of Brady et al.³⁵ Interestingly, incubating dye-APTES labeled silica beads in
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neutral pH conditions at lower concentration ($0.025 \text{ m}^2.\text{mL}^{-1}$) led to a rapid loss of APTES-bound dye molecules, suggesting that dilute conditions favor displacement of the equilibrium towards 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- N_3)-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.

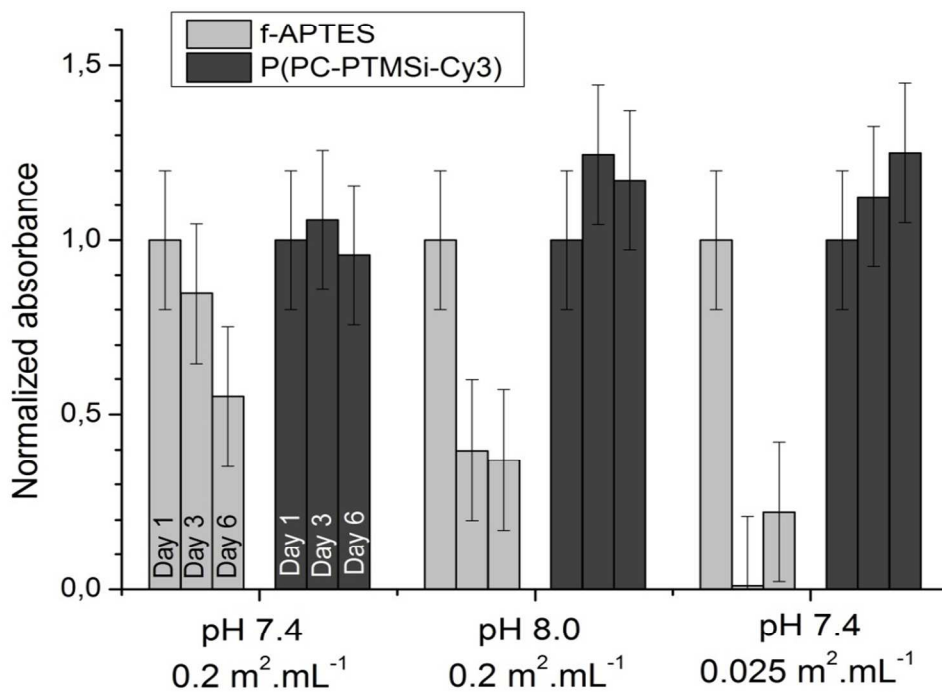


Figure 3: Normalized absorbance values for f-APTES- and P(PC-PTMSi-Cy3)-grafted silica beads at three different pH and concentration conditions. Absorbance values were measured every 3 days at 494 nm (fluorescein) and 570 nm (Cy3) for f-APTES and P(PC-PTMSi-Cy3)

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3 grafted silica beads, respectively. Absorbance values were normalized to 1 at time zero. Error
4 bars were estimated from reproducibility variations, including variations in silica amount after
5 purification, which may account for values slightly above 1 within error bars.
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13 **3.4. Bioconjugation of the clusters.** Cyclooctyne-azide click chemistry was chosen as a
14 versatile bio-orthogonal conjugation technique. To demonstrate the potential of P(PC-PTMSi-
15 N₃)-coated QD-NSCs for biodetection, we conjugated them to streptavidin and tested them on
16 biotinylated substrates as a model targeting system. To this end, streptavidin was functionalized
17 with DBCO, a strained alkyne moiety which enables cyclooctyne-azide coupling without the
18 need for copper (I) catalyst. An NHS-ester – DBCO linker was randomly reacted with some
19 lysines available on the streptavidin surface and the protein was purified by ultrafiltration. A 3:1
20 DBCO-NHS:streptavidin molar ratio was chosen to enable functionalization while keeping a low
21 number of DBCO per streptavidin. Indeed, since we aim at creating a versatile platform, this
22 procedure should be envisioned for reactions such as antibody conjugation and overconjugating
23 antibodies can lead to their loss of functionality. P(PC-PTMSi-N₃)-coated QD-NSC beads were
24 then reacted with DBCO-streptavidin and purified by centrifugation without any appreciable loss
25 of colloidal stability. Microscopic examination of biotinylated agarose beads incubated with
26 these streptavidin-QD-NSCs showed a strong fluorescent signal (Figure 4(A)). By contrast, the
27 interactions of the control, P(PC-PTMSi)-coated QD-NSCs, with biotin-bearing agarose surfaces
28 appear as negligible, a fact that can be attributable to the low-fouling properties of the copolymer
29 (Figure 4(B)). Altogether, these results demonstrate that DBCO-streptavidin binds to the P(PC-
30 PTMSi-N₃)-coated QD-NSC specifically via cyclooctyne-azide conjugation and that the
31 anchored streptavidin retains its biotin-recognition functionality.
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3 An additional control consisting of QD-NSCs mixed with non-DBCO streptavidin also showed
4 very little nonspecific fluorescent signal (Supporting Information, Figure S10). When non-
5 DBCO streptavidin is mixed with the P(PC-PTMSi-N₃)-coated QD-NSCs, some residual non-
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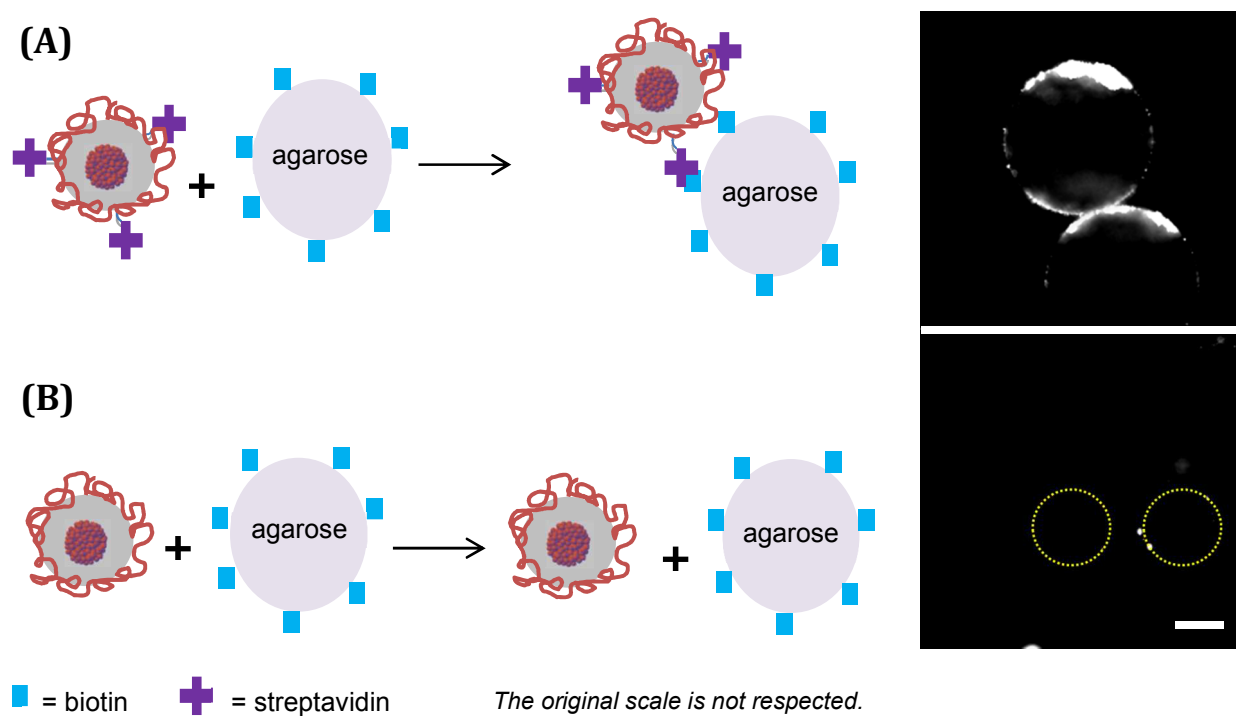


Figure 4: P(PC-TMSi-N₃)-coated QD-NSC beads reacted with streptavidin (A) or as such (B) were left to react for 15 min with biotin agarose beads in HEPES, 10 mM, 150 mM, pH 7.4 and washed thereafter. The epifluorescence microscopy pictures on the right correspond to biotin agarose beads from both experiments, respectively, deposited onto a coverslip. The yellow dotted circle on the bottom picture delimitates the agarose beads, which cannot be seen by

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3 fluorescence. Scale bar= 10 μm
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10 11 **4. CONCLUSION** 12

13 In this work, we presented a multi-step strategy to design colloidal fluorescent nanosized
14 clusters (NSCs) based on silica-embedded quantum dots (QDs). Firstly, QD clusters were formed
15 in a cetyltrimethylammonium bromide-mediated microemulsion and a silica shell was
16 subsequently grown on their surface by a Stöber-inspired process. This reproducible
17 methodology led to compact and very bright clusters of approximately 130 nm diameter.
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20 A particular focus was made on the surface chemistry tailoring of QD-NSCs with the synthesis
21 and characterization of an original multidentate polymer-silane hybrid. After this polymer-silane
22 hybrid was grafted on the surface of Stöber silica beads, we demonstrated that its zwitterionic
23 chain was indeed responsible for a low-fouling effect with regards to a protein such as bovine
24 serum albumine and for the colloidal stability of copolymer-capped silica beads in a
25 physiological buffer. Moreover, the multiple silane anchoring functions of the polymer-silane
26 hybrid seemed to contribute to its better anchoring on the surface of silica beads when compared
27 to individual silane chains, which were more prone to unbinding over time due to hydrolysis.
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30 After the polymer-silane hybrid was grafted onto the silica shell of the QD-NSCs, its azide
31 moiety was effectively reacted with a cyclooctyne-functionalized protein as demonstrated by a
32 specific biomolecular affinity test.
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35 The nanobeads that have been designed constitute a versatile biosensing tool that can be
36 tailored beyond the proof-of-concept showed in this paper e.g. by changing the nature of the
37 inorganic particles encapsulated or the chemical functions available on the surface. Taken
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3 altogether, the results obtained provide interesting perspectives in terms of applications, for
4 example as ultrasensitive single-molecule detection probes within complex biological samples or
5 as signal amplifiers in rare target detection.
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10 11 12 ASSOCIATED CONTENT

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16 Synthesis and size characterization of bare silica beads, synthesis of QDs, photoluminescence
17 spectrum for QDs and QDs-NSCs, synthesis and characterization of the *N*-(11-azido-3,6,9-
18 trioxaundecan)methacrylamide (IR Spectroscopy, ¹H NMR and ¹³C NMR in CDCl₃), reaction
19 scheme for the synthesis of P(PC-PTMSi-N₃), additional characterization of P(PC-PTMSi-N₃)
20 (IR Spectroscopy, ¹H NMR), TGA results, intensity-weighted size distribution obtained from
21 dynamic light scattering at one angle for P(PC-PTMSi)-coated silica beads in HEPES/NaCl
22 buffer, additional control experiment to the bioconjugation experiment with STA without a
23 dibenzocyclooctyne moiety. (PDF)
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35 36 AUTHOR INFORMATION

37 38 **Corresponding Author**

39
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41 *E-mail: thomas.pons@espci.fr
42
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44 45 **Present Addresses**

46
47 † UMR 8638 CNRS, Faculté de Pharmacie de Paris, Université Paris Descartes, Sorbonne Paris
48 Cité, 4 avenue de l'observatoire, 75006 Paris, France.
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52 53 **Author Contributions**

1
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3 The manuscript was written through contributions of all authors. All authors have given approval
4 to the final version of the manuscript.
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19 Paris, France) for the sharing of their equipment.
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