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Hybrid gold nanoparticle–quantum dot self-assembled nanostructures driven by complementary artificial proteins†

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Hybrid nanostructures are constructed by the direct coupling of fluorescent quantum dots and plasmonic gold nanoparticles. Self-assembly is directed by the strong affinity between two artificial α -repeat proteins that are introduced in the capping layers of the nanoparticles at a controlled surface density. The proteins have been engineered to exhibit a high mutual affinity, corresponding to a dissociation constant in the nanomolar range, towards the protein-functionalized quantum dots and gold nanoparticles. Protein-mediated self-assembly is evidenced by surface plasmon resonance and gel electrophoresis. The size and the structure of colloidal superstructures of complementary nanoparticles are analyzed by transmission electron microscopy and small angle X-ray scattering. The size of the superstructures is determined by the number of proteins per nanoparticle. The well-defined geometry of the rigid protein complex sets a highly uniform interparticle distance of 8 nm that affects the emission properties of the quantum dots in the hybrid ensembles. Our results open the route to the design of hybrid emitter–plasmon colloidal assemblies with controlled near-field coupling and better optical response.

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Introduction

Tailoring the properties of light down to the nanometer scale has recently benefited from two major advances: the engineering of collective oscillations of free electrons in noble metals, surface plasmons, and the quantum confinement of noble metals and semiconductors leading to robust and efficient fluorophores. Efforts to couple the field enhancement and confinement near metal nanoparticles and the highly designable fluorescence of quantum dots (QD) within hybrid nanostructures has opened new horizons for nanophotonics^{1,2} with

promising applications in biomedical diagnosis (imaging and sensing)^{3,4} and therapy (optical hyperthermia),^{5,6} energy saving (reduced heat production in electronic chips)⁷ and green production (enhanced photovoltaics).⁸ However, the way leading to these objectives is still facing two major challenges: the preparation of optically activated metallic and semiconductor nanoparticles (NP) of controlled size, crystallinity and morphology and their organization into 2D and 3D higher-order architectures of well-controlled topology for functional devices.

The optoelectronic properties of these self-assembled hybrid QD–metal nanoparticle structures derive from a range of near-field mechanisms including energy transfer (*e.g.* Fluorescence Resonance Energy Transfer, FRET) and field enhancement (*e.g.* Surface-Enhanced Raman Scattering, SERS and Metal-Enhanced Fluorescence, MEF) that are directly affected by the structural and electrostatic parameters of self-assembly such as interparticle Coulomb interactions, dielectric constant of the material, topology and morphology of the assembly and its constituents. Among these parameters, the interparticle distance has a tremendous effect on the optical coupling of QDs with neighbouring nanoparticles within higher-order self-assembled architectures. In particular, the photoluminescence intensity of QDs is enhanced,⁹ diminished¹⁰ or shifted⁹ due to the exciton coupling between identi-

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cal proximal QDs or channelled towards other QDs if the emission band of one type overlaps the absorption band of the other.¹¹ When QDs are coupled to metal plasmonic nanoparticles, the exciton–plasmon coupling strongly depends on the interparticle distance in the NP assemblies and the energy overlap between plasmon and exciton bands.^{9,12,13} The QD photoluminescence intensity can be either enhanced or attenuated due to the competition between non-radiative energy transfer to plasmonic NPs and the local electric field enhancement.^{2,12,13} QD–metal hybrid nanostructures have been prepared using DNA strands,^{13,14} antibodies¹⁵ and proteins such as BSA^{16,17} or streptavidin^{15,18–20} which offer new opportunities to tailor the interparticle distance or the self-assembly structure.

In this article, we induce specific assembly by using one representative of highly specific artificial protein pairs that is created by choosing one specific protein and selecting the pair-forming partner by directed evolution.^{21,22} The family of artificial repeat proteins named α Reps are efficiently produced as recombinant proteins with a very stable folded structure and can be easily tailored to be grafted onto metallic surfaces using metal-binding cysteine tags. Furthermore, a very large library of α Reps with a variable surface was built and this allows the identification of new proteins binding tightly and specifically to any target protein of interest²³ using directed evolution methods.^{21,22} A specific pair of α Reps named A3/ α 2 obtained previously was chosen here for its small and compact complex prone to generate 3–10 nm interparticle gaps and for its facile grafting onto metallic and semiconductor surfaces in order to drive the QD–QD and QD–metallic gold nanoparticle (AuNP) self-organization. We have recently demonstrated the principle of colloidal self-assembly driven by pairs of α -Rep proteins for homogeneous ensembles of protein-capped gold nanoparticles (AuNPs).²⁴ On the other hand, we have shown how to prepare stable bioactivated QDs with high affinity hydrophilic ligands,²⁵ that are being transposed here to A3 and α 2 proteins. Each complementary protein is tagged with a tri-cysteine tag motif at its C-terminal end and prepared at the milligram scale from small scale *E. coli* bacterial cultures.²¹ Next, the proteins are grafted onto peptide-stabilized CdSe@ZnS core–shell QDs by ligand exchange. The efficiency of protein grafting on the QD surface is demonstrated by the induction of the self-assembly of complementary α Rep protein-grafted QDs driven by the A3– α 2 protein affinity. Finally, our method is successfully applied to the self-assembly of hybrid QD–AuNPs from mixtures of complementary α Rep protein-grafted AuNPs and QDs. The extent and size of the superstructures depend on the molar ratio between the proteins and the nanoparticles used in the ligand exchange step. SAXS and TEM analysis of both QD–QD and QD–AuNP self-assembled ensembles reveal a highly uniform interparticle distance which is a crucial parameter to reduce the inhomogeneous dispersion of the optical behaviour of the hybrid superstructures. Finally their optical properties suggest that protein pairing affects the exciton or plasmon–exciton coupling within these nanostructures.

Experiment

Materials

Hydrogen tetrachloroaurate(III) trihydrate (HAuCl₄) and tetramethylammonium hydroxide, 25% w/w aqueous solution (TMAOH), were purchased from Alfa Aesar; tri-sodium citrate dihydrate and chloroform were purchased from Carlo Erba Reagents; DL-dithiothreitol, gold nanoparticles (10 nm diameter, OD 1, stabilized suspension in 0.1 mM PBS, reactant free), agarose, sodium phosphate monobasic (NaH₂PO₄), sodium chloride (NaCl) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich. Boric acid was purchased from Honeywell Fluka and Cys-Cys-Cys-11-aminoundecanoyl-15-amino-4,7,10,13-tetraoxapentadecanoyl was purchased from PolyPeptide Laboratories or Agentide Inc. Proteins were stored at –80 °C in pH 7.4 phosphate buffer saline. Ultrapure Millipore water (18.2 M Ω) was used. Formvar/carbon film 300 mesh nickel grids were purchased from Oxford Instruments. All glassware was washed with aqua regia and rinsed with ultrapure water and ethanol. UV/Vis spectroscopic measurements were carried out on a Cary-100 UV–vis NIR spectrophotometer using Carry UV Win (version 3.00) software. Emission spectra were recorded on a Fluorolog-3 fluorimeter (Horiba Jobin–Yvon) using FluorEssence (version 2.0.9.0).

Peptide grafting for the stabilization of AuNPs and QDs

Gold nanoparticle preparation. 5 mL of 11 nM commercial suspension of 10 nm Au nanoparticles or 16 nm homemade citrate-capped nanoparticles²⁶ were incubated with an aqueous solution of Cys-Cys-Cys-11-aminoundecanoyl-15-amino-4,7,10,13-tetraoxapentadecanoyl (C5PEG4, 7 μ L, 20 mM). The mixture was allowed to stand for 2 h and is then washed, filtered and concentrated using Amicon® Ultra – 0.5 mL 100 K centrifugation filters and 20 mM NaP pH 7.4 buffer through multiple centrifugation cycles (3800g, 2 min). Typically, 230 μ L of 180 nM AuNP–peptide suspension was obtained.

Quantum dot preparation. The QDs used in this study were commercial CdSe@ZnS core@shell nanoprisms (Qdot™ 655 ITK™ organic quantum dots, Thermo Fisher Scientific) when no indication is given. Otherwise nanospheres (Qdot™ 545 ITK™, Thermo Fisher Scientific) were also used for comparison. Water solubilization and stabilization of quantum dots were achieved according to a previously described method.²⁷ First, 1 mL of 1 μ M commercial suspension of red quantum dots in decane was flocculated by centrifugation to transfer the nanocrystals from decane to chloroform. For this purpose, 1 mL of the quantum dot suspension was separated into 5 microtubes with 800 μ L of a 75/25 methanol/isopropanol mixture and was then centrifuged (660g, 5 minutes). The solvent was removed and the flocculated nanocrystals were redispersed in 1 mL of chloroform. This organic suspension was mixed successively with 70 μ L of 20 mM aqueous C5PEG4 solution and 12 μ L of TMAOH at 25% (w/w) in water. These additions induced the transfer of the nanocrystals from the organic solvent to the aqueous phase. Chloroform was

removed after 15 min of incubation and the mixture was evaporated (60 mbar, 20 min, room temperature) to remove the organic solvent traces. The excess peptide molecules were removed by size exclusion chromatography using Sephadex G-25 columns (NAP-5 from GE Healthcare) equilibrated and eluted with 20 mM NaP pH 7.4 buffer. The obtained aqueous suspension of QDs had a concentration of 500 nM.

Protein conjugation on AuNPs and QDs

First, cysteine-tagged α Rep proteins were incubated at 12 °C for 2 h in 100 mM DTT in order to reduce their intra- and inter-protein disulfide bonds. The excess DTT was removed by centrifugal elution through desalting columns (Zeba Spin Desalting Columns, 7 K MWCO) with 20 mM NaP pH 7.4 buffer. Next, peptide-stabilized nanoparticles were functionalized by incubating them (overnight, 12 °C) with 100 and 30 molar equivalents of proteins (α 2 or A3) for QDs and AuNPs, respectively. After incubation, the excess protein was removed by using an ultrafiltration unit Amicon® Ultra – 0.5 mL with a 30 000 molecular weight cutoff (MWCO) at 7200 rpm for 2 min and by washing 5 times with 20 mM NaP pH 7.4 buffer. The purified QD–protein and AuNP–protein conjugates were finally redispersed in 20 mM NaP pH 7.4 buffer.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to monitor the charge and size variations in semiconductor and gold nanoparticles using a Gel XL Ultra V-2 (Labnet International, Inc., USA) horizontal electrophoresis system. The agarose gel was prepared by dissolving agarose (500 mg) in 20 mM borate pH 8 buffer (100 mL) at 90 °C. This solution was then poured into four 53 mm \times 53 mm gel trays or two 130 mm \times 59 mm trays within a gel caster. The gel was run in 20 mM borate pH 8 buffer for 20 to 45 minutes at a voltage of 9 V per 1 cm of gel and observed under UV light (365 nm).

A3/AuNP and A3/QD stoichiometry determination

AuNP-A3 and QD-A3 were prepared and purified from the excess A3 protein according to the methods described above. The concentrations of Au NPs and QDs were then determined by ICP-MS measurements. The A3/AuNP and A3/QD stoichiometries were determined by fluorescence emission measurements after dissolving the inorganic core of the nanoparticles while preserving the proteins as follows. The dissolution of the AuNPs was realized by incubating 32 μ L AuNP-A3 (90 nM) with 16 μ L saturated KCN for 1 hour. The dissolution of QD-A3 was realized by incubation with acidic HCl solution for 12 hours according to the literature.²⁸ Standard A3 solutions at different concentrations from a stock solution (concentration 37 μ M) were prepared in Na phosphate buffer (pH 7.4) and the fluorescence intensity *vs.* concentration was measured. According to the standard curve, the A3 concentration was determined by measuring the fluorescence intensities of the solution resulting from the dissolution of AuNP-A3 or QD-A3.

Transmission electron microscopy (TEM)

The 300 mesh carbon coated nickel grids were placed for 1 min on top of a 40 μ L sample droplet and dried up with paper. Examination was performed using a JEOL 1400 transmission electron microscope operated at 120 kV and supplied with a GATAN Orius 1000 camera. Particle sizes and interparticle distances were determined from TEM micrographs using the ImageJ Software.

Surface plasmon resonance (SPR)

Binding experiments and kinetic assays were carried out at 25 °C using a BIACORE 3000 (GE Healthcare) of the Platform of Molecular Interactions of the Institute of Biology Paris Seine (IBPS, Sorbonne University). α Rep proteins and BSA were immobilized on the CM5 sensor-chip (carboxymethylated dextran) through covalent amidation to the primary amine groups. For the immobilization step, the solutions of α 2 and A3 (200 nM) and a solution of 50 μ g ml⁻¹ of BSA, prepared in 10 mM sodium acetate buffer adjusted to a pH adapted to the pI of each protein (pH 5.0 for A3, pH 4.5 for BSA and pH 5.5 for α 2), were injected at 10 mL min⁻¹. A contact time of 7 min was used to obtain a high level of immobilization quantified as follows: 3900 RU (Resonance Units), 11 600 RU and 1500 RU for A3 on flow cell two (FC2), BSA on FC3 and α 2 on FC4 respectively. FC1 was left blank as a reference surface for non-specific binding and refractive index variations. This immobilization step was followed by the injection of 1 M ethanolamine HCl pH 8.4 to saturate the free residual amine groups. For binding experiments, a 2.3 nM solution of QD- α 2 in HBS-EP buffer (GE Healthcare, 10 mM pH 7.4 HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% P20) was flown over FC1 for the blank, FC2 for A3, FC3 for BSA and FC4 for α 2 at 5 μ L min⁻¹ for 5 min. Kinetic measurements were performed by injecting several concentrations of α 2 (between 0 and 2.4 nM) over the A3 surface at a flow rate of 5 μ L min⁻¹ with a 5 min association phase and an 8 min dissociation phase corresponding to the injection of the buffer solution. After each assay, the sensor-chip was regenerated by performing one injection of glycine-HCl buffer (pH 1.5) at 30 μ L min⁻¹ for 30 seconds or two injections of glycine-HCl buffer (pH 2.0) at 30 μ L min⁻¹ for 30 seconds. All experiments were performed in triplicate.

Preparation of nanoparticle mixtures

For the preparation of QD-QD or hybrid QD-AuNP self-assembled nanostructures, suspensions of freshly prepared QD–protein or AuNP–protein conjugates were used at the same concentration: 96 nM for QD-AuNPs or 100 nM for QD-QDs. Mixtures of these complementary populations were prepared with a final volume of 12 μ L for QD-AuNPs or 30 μ L for QD-QDs, adjusting volumes of each suspension to obtain particle- α 2 : particle-A3 volume ratios of 5 : 1, 2 : 1, 1 : 1, 1 : 2, and 1 : 5. The same procedure was applied to the preparation of QD- α 2/AuNP- α 2 non-specific mixtures and competition mixtures, where the QD-A3/AuNP- α 2 mixtures were incubated with an excess of free α 2 protein or free A3 protein (adding

100 molar equivalents) for a competition test. The mixtures were gently agitated before their incubation for 12 hours at 12 °C. Finally an excess of free protein was added after incubation on the QD-QD or hybrid QD-AuNP self-assembled nanostructures for a reversibility test.

Nanoparticle concentration determination

The concentrations of QDs in suspensions were calculated as described by Jasieniak *et al.*,²⁹ relying on the first absorption peak energy (E_{1S}) in order to calculate the QD absorption coefficient

$$\epsilon_{1S} = 155\,507 + 6.67054 \times 10^{13} \times e^{-E_{1S}/0.10551}.$$

QD concentration was then determined by using the absorbance of the first absorption peak and its half-width-half-maximum, allowing a rescaling of the concentration from the nominal size distribution of the samples. The AuNP concentration was calculated from their absorption coefficient at 520 nm: $\ln \epsilon_{\lambda_{LSP}} = 3.32111 \times \ln D + 10.80505$, where D is the core diameter of the gold nanoparticles measured by TEM, according to Liu *et al.*³⁰

Small angle X-ray scattering (SAXS)

Small angle X-ray scattering images were obtained with the Pilatus 300k detector (dectris) mounted on a home-made Guinier setup equipped with a $\text{CuK}\alpha$ microsource ($\lambda = 1.541 \text{ \AA}$) from Xenocs. The sample to detector distance (277 mm) has been calibrated by using silver behenate. The X-ray patterns were therefore recorded for a range of reciprocal spacing $q = 4\pi \sin \theta / \lambda$ from 0.01 to 1.75 \AA^{-1} where θ is the diffraction angle. The acquisition time was 1 hour. The scattering intensities as a function of the radial wave vector were determined by circular integration.¹⁰

Results and discussion

QD-protein and AuNP-protein dispersions are prepared by a ligand exchange process in the presence of excess protein, as shown in Fig. 1a. The morphological and optical properties of the CdSe@ZnS core@shell quantum dots and the gold nanoparticles are summarized in Fig. S1 and S2, respectively.† The AuNPs and QDs have average diameters of $8.0 \pm 0.9 \text{ nm}$ and $8.9 \pm 1.4 \text{ nm}$ respectively. Their colloidal stability is maintained in buffered medium at pH 7.4 using an optimized tri-cystein PEGylated ligand.^{27,31} The 8 nm diameter AuNPs exhibit a localized surface plasmon resonance (LSPR) at 520 nm (black curve) and the QDs exhibit an intense red emission band at 655 nm (red curve).

Upon protein grafting, the fluorescence efficiency of the water-soluble QD-C5PEG4 is reduced by 35% but the emission at 655 nm does not undergo any spectral shift. The AuNP absorption band at 520 nm is fully preserved (Fig. 1c).

The titration of QD-C5PEG4 with an increasing amount of protein has been monitored by agarose gel electrophoresis (Fig. 2). The grafting of proteins onto 9 nm or 2.5 nm QD and

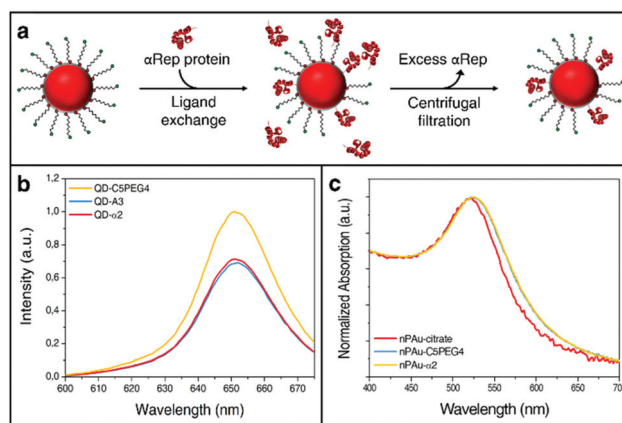


Fig. 1 (a) Schematic representation of protein grafting by ligand exchange at the surface of QD-C5PEG4. (b) Fluorescence emission spectra of peptide-stabilized QDs and protein-grafted QDs in PBS buffer for a 16 nM total QD concentration (excitation wavelength is 350 nm). (c) Normalized UV-visible spectra of the citrate-stabilized AuNPs in water, peptide-stabilized AuNPs and protein-grafted AuNPs in PBS buffer.

8 nm AuNPs is investigated through separate incubations with increasing amounts of proteins which are then compared after a 20 min migration time.³² The migration towards the positively charged electrode indicates that the net charges of peptide-stabilized and protein-grafted nanoparticles are negative. Interestingly, as proteins are gradually introduced into the peptide capping layer, the electrophoretic migration distance becomes shorter, which is consistent with the increase in the overall size of the functionalized particle and the reduction of the net negative charge at pH 8 since the proteins have pIs of 5.43 (A3), 7.07 ($\alpha 2$) and 4.7 for the peptide (C5PEG4). Moreover, the progressive decrease in the migration distance gives rise to a plateau that can be related to the saturation of the surface with a maximum number of α Rep proteins onto the nanoparticle surface leading to the maximum size and minimum net negative charge. In the case of $\alpha 2$ (Fig. 2, left panels), the migration distances are shorter than the corresponding ones for A3, which is attributed to the smaller size and net charge at pH 8 compared to A3.²⁴ Additionally, A3-grafted nanoparticles tend to form short trails in the agarose gel. This can be attributed to the propensity of A3 to dimerize at high concentrations,^{21,24} which favors interparticle interactions within the highly concentrated spot of particles deposited on the gel but can also alter the effective protein/particle stoichiometry if one grafted A3 dimerizes with one free A3.

As summed up in Table 1, the maximum number of proteins grafted onto the NP surface scales up with the surface area of the nanoparticles, A_{NP} .

The proteins can be approximated to two blocks of $(2.8 \times 2.4 \times 4.3 \text{ nm}^3)$ size for $\alpha 2$ and of $(3 \times 3 \times 6 \text{ nm}^3)$ size for A3. As expected from their relative sizes, the corresponding area of each grafted protein, denoted by $A_{\text{prot}}^{\text{NP}}$, decreases with the size of the protein. The area of one grafted A3 protein is found to

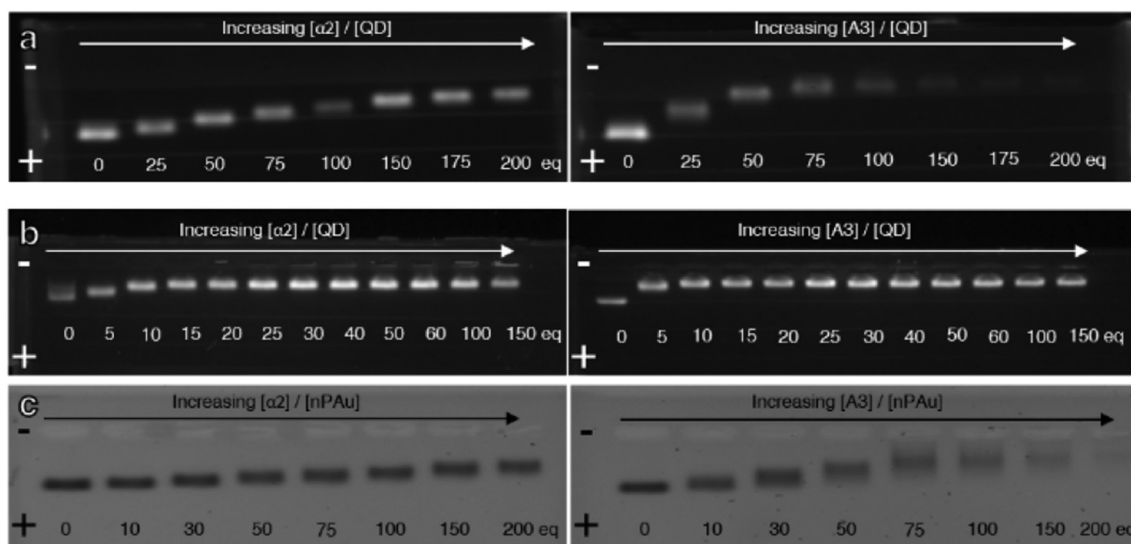


Fig. 2 Agarose gel electrophoresis of peptide-stabilized (a) 9 nm QD⁶⁵⁵, (b) 2.5 nm QD⁵⁴⁵ and (c) 8 nm AuNPs incubated with increasing amounts of $\alpha 2$ (left) or A3 (right). The buffer used is at pH 8.

be around 4 to 5 nm² which is significantly smaller than the expected A3 size (min area of 9 nm²) and therefore is hardly compatible with a compact monolayer of A3 proteins. This observation suggests that surface binding creates an effective high local concentration of A3 leading to the formation of a double layer of A3 as this protein is known to form A3-A3 dimers when concentrated. This does not prevent the subsequent A3- $\alpha 2$ pairing as the latter complex is much more stable.²³ To perform a more quantitative evaluation of the protein/particle stoichiometry, the amount of bound proteins was titrated after dissolution of the inorganic core of the NP-protein conjugates while preserving the proteins by using the intrinsic fluorescence intensity of the A3 protein. In the case of the A3 protein, AuNP-A3 conjugates and QD-A3 were first thoroughly cleared of excess free A3 proteins by multiple filtrations. The concentration of AuNP was first determined by inductively coupled plasma mass spectroscopy (ICP-MS). According to Dyer *et al.* the gold core of the purified AuNP-A3 was then dissolved in 3.7 M KCN, releasing A3 proteins. The concentration of A3 proteins initially bound to the AuNP was determined by fluorescence spectroscopy (Fig. S3[†]). We found that A3-saturated AuNPs, prepared with 100 molar equivalents of A3, contain an average of 80 A3 per AuNP (see Fig. S3[†]), which is in good agreement with the migration saturation estimate (Table 1). In the case of QD-A3, the CdSe@ZnS core shell QDs were dissolved in the presence of an acidic HCl solution according to Debayle *et al.* QD-A3 prepared from 100 molar equivalents of A3 were found to contain an average of 20 A3 per QD. This latter observation is in agreement with a smaller affinity of the A3 protein for the QD surface than for the Au surface (see Fig. S4[†]). Unfortunately, this method cannot be applied to $\alpha 2$ since its intrinsic fluorescence emission is too low to assess the concentration of $\alpha 2$ protein after NP dissolution.

Table 1 Summary of geometrical data of the NP-protein: A^{NP} designs the area of the NPs considered to be a cube, $N_{\alpha 2}^{\text{sat}}$ corresponds to the maximum number of proteins grafted onto the NP surface extracted from the electrophoresis gel when the plateau is reached. $A_{\text{prot}}^{\text{NP}} = A^{\text{NP}} / N_{\text{prot}}^{\text{sat}}$ is the corresponding area for one grafted protein

NP	A^{NP} (nm ²)	$N_{\alpha 2}^{\text{sat}}$	$N_{\text{A3}}^{\text{sat}}$	$A_{\alpha 2}^{\text{NP}}$ (nm ²)	$A_{\text{A3}}^{\text{NP}}$ (nm ²)
8.9 nm QDs	475	$\geq 200 \pm 13$	100 ± 13	$\geq 2.4 \pm 0.2$	4.8 ± 0.6
2.5 nm QDs	38	15 ± 3	10 ± 3	2.5 ± 0.4	3.8 ± 0.9
8 nm AuNPs	384	$\geq 200 \pm 13$	75 ± 13	$\geq 1.9 \pm 0.2$	5.1 ± 0.7

To further assess the specific binding between QD-grafted proteins and complementary proteins, A3 proteins are immobilized on Surface Plasmon Resonance (SPR) sensor chips and subjected first to a high flux of QD- $\alpha 2$ and then to an influx of pure buffer. The SPR sensorgrams are shown in Fig. 3a. A strong positive association phase is observed in the case of the A3 coated surface. When the nanoparticles bound to the A3-coated surface are washed, a reduction in the SPR response is observed indicating that dissociation occurs leading to a new equilibrium state. No association is observed during control experiments performed either by immobilizing the identical $\alpha 2$ protein or Bovine Serum Albumin (BSA). These observations demonstrate the ability of QD- $\alpha 2$ to specifically bind to the A3 protein coated surface. To estimate the affinity constant K_D , the same experiment is performed with different QD- $\alpha 2$ concentrations, as shown in Fig. 3b. The data are fitted with a 1:1 single exponential interaction model (Fig. 3b, black lines) in both association and dissociation phases, suggesting a single binding mechanism of QD- $\alpha 2$ + A3 = QD- $\alpha 2$ -A3. The dissociation constant was extracted from three different approaches detailed in the experimental part and ESI (Fig. S5[†]). All the quantitative analyses consistently

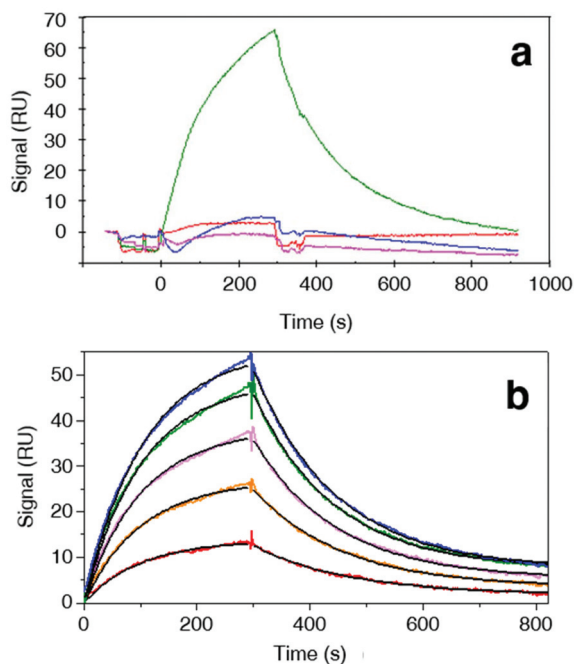


Fig. 3 QD- α 2 affinity for the A3 coated surface. (a) SPR sensorgrams during association and dissociation phases monitoring the interactions between QD- α 2 nanoparticles (2.3 nM) and immobilized protein surfaces: A3 (green curve), bovine serum albumin (blue curve), α 2 (pink curve) and a bare surface (red curve). (b) SPR sensorgrams during association and dissociation phases monitoring the interactions between the A3 immobilized surface and QD- α 2 nanoparticles at different concentrations: 0 nM; 0.4 nM; 0.8 nM; 1.2 nM; 1.6 nM; 2.0 nM and 2.4 nM (from bottom to top). Solid black lines are exponential fits to the association and dissociation phases.

yielded 5 nM dissociation constant similar to the one measured by ITC in free α 2/A3 protein pairs.²²

The protein pair affinity is high enough to drive the self-assembly of protein-grafted QD when the suspensions of QD- α 2 and QD-A3 are incubated together. The electrophoretic migrations of the complementary mixtures of QD- α 2 and QD-A3 (Fig. 4a, tracks iii–vii) are monotonously intermediate between those of pure protein-grafted QDs (Fig. 4a, tracks ii and viii) as the molar ratio between the two populations is varied. The migration distances were found to directly depend on the composition of the mixture. A brighter spot was seen on the same side of the excess partner (top side for tracks iii and iv where QD-A3 is in excess; bottom side for track vii where QD- α 2 is in excess) superimposed with a broader central trail which dominates tracks v and vi. The intermediate migrations observed for the complementary QD mixtures indicate a specific interaction that cannot be accounted for by simple superposition of non-interactive mixtures of the native suspensions. Zeta potential measurements confirm that protein grafting results in a decrease in the net surface charge of the NPs, which is in agreement with the isoelectric points of the proteins (Table S1†). The zeta potentials of the complementary QD mixtures cannot be determined due to the increased polydispersity of the mixtures. Indeed TEM obser-

vations reveal the formation of spatially limited assemblies giving oligomers of nanoparticles that cannot be found in the original QD- α 2 or QD-A3 populations (Fig. 4). The effect of the larger effective size of the complementary QDs appears to be screened by the intermediate net charge which acts as the dominant factor maybe because the electrophoresis is performed with low agarose percentage gel. The statistical analysis of the number of QDs per oligomer reveals the distribution of limited-size oligomers with 2 to 30 QDs per oligomer (see Fig. 4a), in agreement with the relatively low charge of the A3 protein bound to the surface of the QD (20 proteins/QD) estimated from fluorescence titration (see Fig. S6†). These observations confirm that the specific interaction between the complementary QDs results in new superstructures with intermediate net charge and larger effective size that can be maximized at QD-A3 : QD- α 2 molar ratios of 1 : 1 to 1 : 2.³³

Small Angle X-ray Scattering (SAXS) experiments were performed to obtain more quantitative structural information about QD self-assemblies, which are summarized in Fig. 5. Pure QD-A3 and pure QD- α 2 suspensions produce similar featureless SAXS patterns (Fig. 5a, black curve). In contrast, the data of the equimolar QD-A3/QD- α 2 mixture show a marked peak at $q = 0.038 \text{ \AA}^{-1}$ (Fig. 5a, blue curve). After baseline subtraction, this broad scattering is well fitted by a Gaussian function centered at $q_0 = 0.0364 \text{ \AA}^{-1}$ (Fig. 5a, inset). In the Debye-Scherrer theory, this feature is characteristic of a small crystal with a center-to-center particle interdistance $d_{\text{RX}} = 17.2 \text{ nm}$. The full width of the peak Δq_0 of 0.0180 \AA^{-1} yields $N = q_0/\Delta q_0 \sim 2$ QD-proteins, which suggests that this broad scattering originates from a pair correlation function between two QDs and is attributed to the QD-A3- α 2-QD elementary pair.³⁴

The QD diameter determined from TEM images is $8.9 \pm 1.4 \text{ nm}$ (Fig. S2b†), and hence $d_{\text{RX}} = 17.2 \text{ nm}$ yields an interparticle distance of $8.3 \pm 1.4 \text{ nm}$, which is in good agreement with the value of $7.6 \pm 2.6 \text{ nm}$ obtained directly by TEM (Fig. S7†). The gap region between the two adjacent QDs matches precisely the expected size of the A3- α 2 protein complex (Fig. 5b). Hence both TEM and SAXS data strongly support the formation of QD-QD self-assemblies driven by the protein pair complexation. Interestingly, when competition experiments are run by adding an excess of free α 2 protein, the characteristic SAXS peak disappears (Fig. 5a, red curve), as expected from the entropic advantage of the free protein over the particle-bound protein in the binding event.^{23,24}

Beyond homogeneous QD-QD or AuNP-AuNP assemblies, our approach allows the formation of hybrid self-assembled nanostructures that associate QDs and AuNPs with a precise control of the interparticle distance. Both protein-grafted QD and AuNP samples were prepared following the procedure described in Fig. 1. The surface density of proteins is adjusted by using 100 or 30 molar equivalents of proteins during the ligand exchange step. Several mixtures of QD-A3 and AuNP- α 2 with different QD : AuNP molar ratios are produced at a fixed total nanoparticle concentration of 96 nM (see the Experiment section). In Fig. 6, TEM images reveal the formation of a protein-driven hybrid self-assembly where QDs and AuNPs

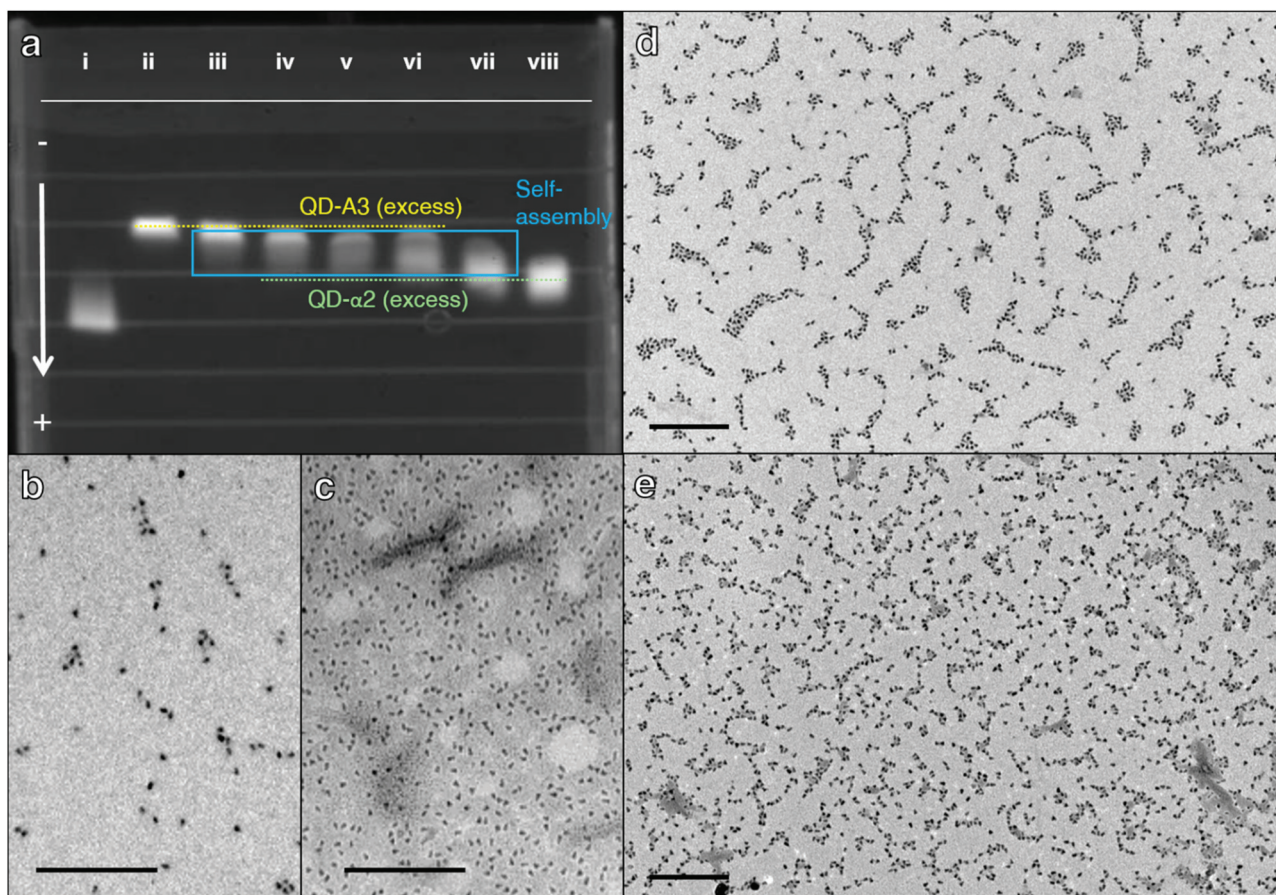


Fig. 4 (a) Fluorescence image of the agarose gel electrophoresis of pure QD-C5PEG4, QD-A3, mixtures of QD-A3 and QD- α 2 at 5 : 1, 2 : 1, 1 : 1, 1 : 2 and 1 : 5 molar ratios respectively and QD- α 2 in tracks i–viii respectively. TEM images of (b) pure QD-A3, (c) pure QD- α 2, (d) 1 : 1 QD- α 2 + QD-A3 mixture and (e) 1 : 5 QD- α 2 + QD-A3 mixture. Scale bars represent 100 nm.

coexist and systematically alternate. Indeed, assemblies of increasing dimensions are observed within the mixtures of complementary A3-grafted QDs and α 2-grafted AuNPs according to the nanoparticle : protein stoichiometry. Hybrid assemblies obtained from QDs and AuNPs with protein-saturated coating are very large (Fig. 6a, Fig. S8 and S9[†]) while mixtures produced from nanoparticles with more moderate surface density of proteins contain much smaller assemblies comprising a few tens of nanoparticles (Fig. 6b and g) down to trimers (Fig. 6c–e and Fig. S10[†]). A closer examination shows that >98% of QDs and >90% of AuNPs are engaged in a self-assembled pair (Table S2[†]), which qualitatively illustrates the high efficiency of the protein affinity driving force in this self-assembly. Control experiments using either a mixture of QD-A3, AuNP- α 2 and an excess of free α 2 proteins (Fig. S11[†]) or a mixture of non-complementary QD- α 2 and AuNP- α 2 (Fig. S12[†]) show dispersed AuNPs and QDs with no significant association.

Small Angle X-ray Scattering (SAXS) experiments performed using a (1 : 1) mixture of QD-A3 and AuNP- α 2 suspensions with protein-saturated coating exhibit a characteristic peak at $q = 0.0395 \text{ \AA}^{-1}$ (Fig. 7, blue curve). After baseline subtraction, this broad scattering is well fitted by a Lorentzian function cen-

tered at $q_0 = 0.0387 \text{ \AA}^{-1}$ (Fig. 7, inset). In the disordered crystal theory of Guinier, the Lorentzian shape indicates a disorder of second kind in a crystal which is liquid-like. This corresponds to the aggregate observed by TEM. The mean center-to-center particle interdistance $d_{\text{RX}}^{\text{QD-AuNP}} = 16.2 \text{ nm}$. In contrast, pure QD-A3 (black curve) and AuNP- α 2 (yellow curve) suspensions as well as the control competition (red curve) and reversibility (green curve) experiments with an equimolar QD-A3/AuNP- α 2 mixture produce featureless SAXS patterns (Fig. 7). A surface-to-surface interdistance of $7.75 \pm 1.15 \text{ nm}$ is estimated from the QD-Au center-to-center interdistance $d_{\text{RX}}^{\text{QD-AuNP}}$ and the NP diameters ($d_{\text{TEM}}^{\text{Au}} = 8 \text{ nm}$ and $d_{\text{TEM}}^{\text{QD}} = 8.9 \text{ nm}$) (see Fig. 7). Additional SAXS experiments performed with different sized QDs (see Fig. S13[†]) and the same AuNPs reveal the same behaviour and confirm a very well-defined interdistance. A surface-to-surface distance of $9 \pm 1 \text{ nm}$ for QD⁶⁰⁵ and $8.75 \pm 1 \text{ nm}$ for QD⁵⁴⁵ is estimated from the center-to-center distances found by SAXS for green and orange QDs (see Fig. S14[†]). As in the case of QD-QD superstructures, this value around 8 nm is in the range of the A3- α 2 protein complex size.

Both SAXS and TEM experiments confirm that complementary and available surface-tethered proteins are necessary to

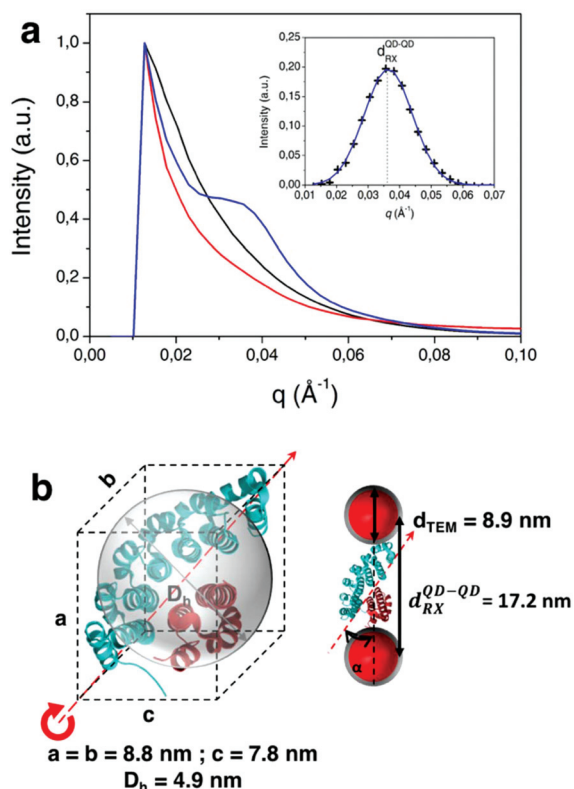


Fig. 5 (a) SAXS data of QD-A3 (black curve) showing the characteristic self-assembly signal when mixed with complementary QD- α 2 (blue curve) and competition experiment run with an excess of free α 2 protein (red curve). Inset: peaks of the QD-A3/QD- α 2 mixture and Gaussian fit (see the text). (b) (Left) Crystallographic data (a , b , c)²⁰ and the hydrodynamic diameter D_h of the A3- α 2 protein complex; (right) Schematic view of the complementary QD arrangement including the QD diameter measured by TEM (d_{TEM}) and the SAXS interdistance measured by SAXS (d_{RX}).

induce the colloidal assembly. The mean surface-to-surface interdistance in the complementary mixtures found by SAXS is 8 nm, in agreement with the protein complex size.

In addition, the protein-driven assembly has a marked effect on the exciton-plasmon interaction inside the hybrid aggregates as illustrated in Fig. 8 and S15.† When a suspension of QD-A3 is mixed with increasing amounts of non-complementary AuNP-A3 (Fig. 8, grey bars), a gradual decrease in the intensity is observed, which can be almost entirely ascribed to the relative dilution and some A3 dimerization at a high AuNP-A3 molar fraction. When the AuNPs are functionalized with α 2 (Fig. 8, red bars), specific complementary interactions induce a much larger quenching of luminescence, which is essentially suppressed for an equimolar mixture, *i.e.* when all QDs and AuNPs are potentially engaged in hybrid superstructures. The sub-10 nm interparticle distance seems to favor the QD (donor) to AuNP (acceptor) energy transfer and non-radiative decay over metal-enhanced fluorescence.^{35,36} Interestingly, the effect due to the modification of the local dielectric constant in the self-assembled structure can be disregarded by comparing the emission of QD-A3- α 2-AuNP mix-

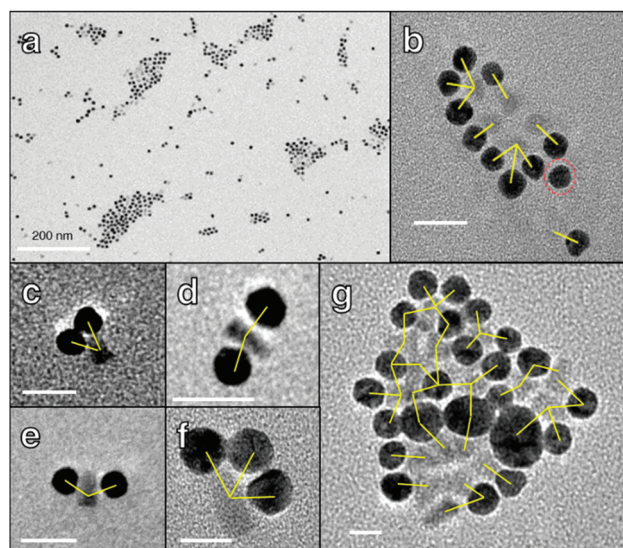


Fig. 6 TEM images of (a) large self-assemblies of QD-A3 and AuNP- α 2 and (b–g) spatially limited self-assemblies QD-A3 and AuNP- α 2. Scale bar is 20 nm for (b) and (d). Scale bar is 10 nm for (c). Yellow bars represent QD-AuNP pairs that are possibly linked by protein pairs. The red dotted circle indicates AuNPs with no obvious link to QDs.

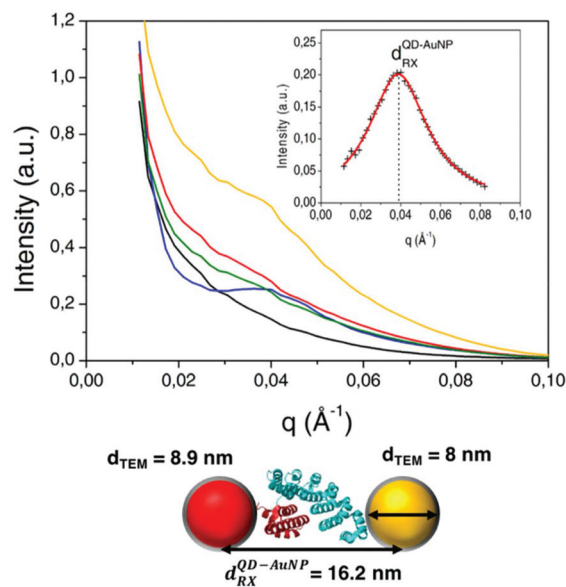


Fig. 7 SAXS data showing a characteristic self-assembly signal when QD-A3 and AuNP- α 2 are mixed together (blue curve). Inset: peaks of the QD-A3/AuNP- α 2 mixture and Lorentzian fit (see text). Pure QD-A3 (black curve) and AuNP- α 2 (yellow curve) as well as competition experiments run with an excess of free α 2 proteins (red curve) and reversibility control experiment performed by the addition of free α 2 proteins (green curve) show no sign of organization. Schematic view of the complementary QD/AuNP arrangement including QD and Au diameters measured by TEM and the center-to-center interdistance ($d_{\text{RX}}^{\text{QD-AuNP}}$).

tures to the equivalently specific but plasmon-free QD-A3- α 2-QD case (Fig. 8, black circles). In the latter case, the luminescence decreases moderately as QD- α 2 are introduced and

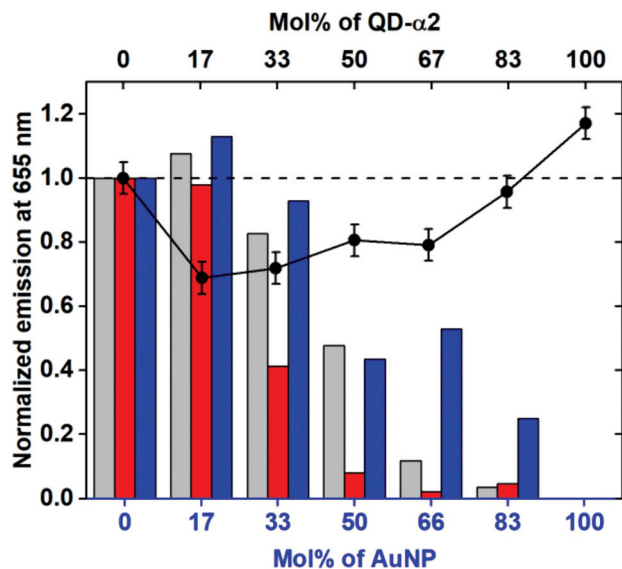


Fig. 8 Histograms showing the evolution of QD-A3 fluorescence ($\lambda_{em} = 655$ nm) in the presence of AuNPs under non-specific (AuNP-A3, grey), specific (AuNP- $\alpha 2$, red) and competitive (AuNP- $\alpha 2$ and excess $\alpha 2$, blue) conditions. Black circle data show the QD-A3 fluorescence ($\lambda_{em} = 655$ nm) in the presence of specific QD- $\alpha 2$ using the same A3- $\alpha 2$ protein pair. Fluorescence intensities are normalized to the value for pure QD-A3 (0 Mol% of AuNP or QD- $\alpha 2$) by taking into account the dilution factor. The dashed line corresponds to the expected normalized fluorescence intensity for QD-A3 corrected based on the dilution.

form larger aggregates but it remains higher than 70% of the intensity value for pure QD-A3 and it increased back as soon as QD- $\alpha 2$ is in excess. The reduction in QD fluorescence observed in the hybrid assembly cannot be solely ascribed to the assembly process or to the modification of the scattering properties by an effective increase in the local dielectric constant but might also be related to the plasmon-mediated non-radiative decay. Finally, when the protein-driven colloidal assembly is performed in the presence of a large excess of free $\alpha 2$ protein, a competition is set for the $\alpha 2$ -A3 protein pair. It is expected that the small $\alpha 2$ protein will preferentially bind to QD-A3 resulting in a mixture where QD-A3- $\alpha 2$ coexists with AuNP- $\alpha 2$ with minimal interaction. The fluorescence of the mixture under competitive conditions (Fig. 8, blue bars) is indeed equal or higher than the fluorescence of the non-specific mixture. In particular, when the AuNP molar fraction is high, the binding of free $\alpha 2$ to form QD-A3- $\alpha 2$ seems to further limit the non-specific binding (A3 dimerization), and the evolution of luminescence is closer to the linear decrease expected for simple dilution. The evolution of fluorescence in complementary interacting QD-A3 (with different emission wavelengths) and AuNP- $\alpha 2$ mixtures also exhibits a quenching as shown in Fig. S15.† However, there is no simple correlation between the spectral overlap of the emission band and the plasmon band that would permit the discrimination of the different contributions of the mechanisms (MET or FRET) involved in the observed coupling (see Fig. S16†).

Conclusion

To conclude, we have shown that high affinity pairs of artificial repeat proteins offer a unique platform to translate specific biomolecular interactions to functional colloidal self-assemblies. The main assets of our approach exploit (i) the very low (around 5 nM) dissociation constant, (ii) the pair specificity that makes it possible to develop, at will, orthogonal pairs for complex hybrid constructions and (iii) the rigid structures of the proteins and their complexes that enable a strategy for topologically determined 3D assemblies. In this work, we focus on creating and characterizing superstructures comprising protein-coated semi-conductor QDs only and their plasmonic hybrid extension by coupling to gold nanoparticles. Using gel electrophoresis and SPR measurements, we demonstrate a generic approach for grafting any alpha-repeat protein on the surface of quantum dots without altering the biorecognition. The self-assemblies have been structurally analysed by TEM and SAXS, which reveal that the surface-to-surface nanoparticle interdistance is around 8 nm which is in the range of the size of the protein pair. As the 3D structure of the A3- $\alpha 2$ complex is known, one can modulate the interdistance between anchored nanoparticles and thus their optical coupling by extending the proteins with additional motifs without destabilizing the binding interface with $\alpha 2$. Given their modular nature and the geometry of the $\alpha 2$ helix arrangement, alphaRep proteins could be engineered either by changing the number of motifs along a chosen protein, each motif resulting in an additional 1 nm distance. We also observe that the extent of the superstructures can be tuned by adjusting the surface density of grafted proteins. The higher the protein:nanoparticle stoichiometry, the larger is the size of the resulting self-assembled superstructure. Conversely, QD-QD or hybrid QD-Au oligomers form when the nanoparticles carry a few bound proteins, giving access to model systems such as QD-Au-QD trimers.³⁷ Finally, the luminescence of the QDs is quenched when they are bound to Au nanoparticles through the A3- $\alpha 2$ bridge. Hybrid superstructures with adjustable luminescence properties should be produced by tuning the spectral overlap between the excitonic and plasmonic resonances making our approach a versatile tool for biosensing applications.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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