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Probing the interactions between disulfide-based ligands and gold nanoparticles using a functionalised fluorescent perylene-monoimide dye†‡

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The binding of disulfides to gold nanoparticles was investigated using fluorescence spectroscopy and a perylene-monoimide dye coupled to a dissymmetric disulfide *via* a tetraethyleneglycolalkyl chain (PMImSS). Quantum chemical calculations using the polarizable continuum model (PCM) predict a strong quenching of perylene-monoimide fluorescence by gold nanoparticles as a result of efficient excitation energy transfer from the dye to the particle. Such quenching is indeed observed when unfunctionalised gold nanoparticles are added to a solution of PMImSS. The fluorimetric titration curves show behaviour indicative of the existence of an equilibrium between free and bound ligands (association constant $5 \times 10^5 \text{ M}^{-1}$), whereas the affinity of thiols and disulfide for gold surfaces is in general assumed to be much higher. Gold nanoparticles fully functionalised with PMImSS were synthesised and purified. Fluorescence correlation spectroscopy shows the appearance of free PMImSS ligands in dilute (approx. pM) suspensions of these PMImSS-functionalised nanoparticles over a period of several days.

Introduction

The photophysics of molecular systems are influenced by their environment, which may be a solvent or another type of embedding matrix. Solvent effects¹ on such fundamental processes as light absorption and emission,² excitation energy transfer and electron transfer^{3–5} are well studied. These effects may partially come from specific interactions between the chromophore and the surrounding matrix molecules, for example through hydrogen bonding, but an important contribution comes from embedding the chromophore in a cavity in a polarizable dielectric medium.

In addition to understanding molecular photophysics in anisotropic, unstructured media such as solvents, glasses and polymers, interest has also developed on finding out what happens when a chromophore is placed in an anisotropic, structured environment, such as near a dielectric interface or a mirror,⁶ or in a photonic crystal⁷ which can enhance or suppress radiative transitions. Particularly intriguing are the effects of noble metal structures

on the photophysics of nearby molecules, exemplified by surface-enhanced Raman scattering^{8,9} and enhancement of the brightness of luminescence^{10–12} or the rate of photopolymerisation.¹³ These phenomena stem from the interaction between the chromophores and collective excitations of the conduction electrons near the surface of noble metals (the surface plasmon polaritons). A tempting objective is to find ways of optimising metal nanostructures in such a way that specific molecular photonic signals are enhanced, as this opens perspectives for ultrasensitive detection in various applications.

The rich surface chemistry of thiols and disulfides combined with colloidal gold particles^{14,15} enables the construction, through self-assembly, of multifunctional nano-objects that can incorporate organic chromophores.^{15,16} One family of chromophores that has been intensively used in such work are the derivatives of fluorescein, in particular adducts of fluorescein isothiocyanate (FITC).^{17–20} Indeed FITC and its precursor aminofluorescein are convenient, well-known, commercially available and inexpensive reactive dyes that can be used as photoactive ‘modules’²¹ in molecular systems. Fluorescein, however, has a limited photostability.²² Moreover, the adducts of FITC have complex photophysics, and at any given pH multiple emitting species contribute to the fluorescence.²³ This gives FITC adducts a particular sensitivity to their environment, and precludes quantitative assessment of fluorescence enhancement or quenching.

Perylene and its derivatives, in particular the perylene imides, are known to be highly fluorescent, photostable dyes. Their rigid structures enable the use of perylene²⁴ and perylene monoimide (PMI) derivatives^{5,25–28} in single molecule spectroscopy. PMI derivatives have recently been used in membrane imaging.²⁹ Derivatives of perylene may therefore be interesting dyes for inclusion as fluorescent modules in molecular assemblies^{30–32} and hybrid nano-objects.

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Here we investigate a disulfide derivative of PMI and its use as a fluorescent probe for the binding of disulfides to spherical gold particles of 5–15 nm diameter (Fig. 1). Such a probe will enable us to investigate the quenching of molecular fluorescence by colloidal gold particles. In order to reliably probe the interaction of the disulfide with the particles and the ensuing fluorescence quenching, the fluorescent dye should not aggregate, have a high fluorescent quantum yield, and exist as a single emitting species in solution. This latter property is not fulfilled by FITC derivatives, as mentioned earlier.

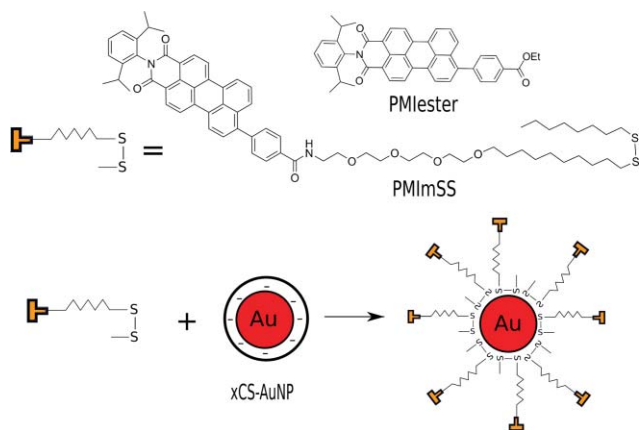


Fig. 1 Molecular structures of model chromophore PMIester and ligand PMImSS. Cartoon of the formation of self-assembled monolayers of PMImSS on the surface of gold nanoparticles.

We first describe quantum chemical predictions of the photophysics of the PMI dye using the polarizable continuum model (PCM).³³ PCM has been formulated to describe the effect of solvents on molecular photophysics, and has successively been modified to include metal particles as part of the dielectric environment of chromophores.^{34,35} We study the absorption and the fluorescence of the model chromophore PMIester and the ligand PMImSS alone in solution, before carrying out fluorescence titrations on the interaction of PMImSS with excess-citrate stabilised gold nanoparticles (xCS-AuNPs).

Finally, we will demonstrate spontaneous ligand desorption from purified, fully functionalised PMImSS-AuNP in dilute solution using fluorescence correlation spectroscopy (FCS). In FCS, a small observation volume (sub-femtolitre) is defined optically by focusing excitation light into the solution through a microscope objective and detecting the fluorescence coming from the observation volume through a confocal pinhole. At sufficiently low concentrations (sub-nanomolar), the focal observation volume will contain on average one molecule or less. The recorded fluorescence signal will show strong fluctuations (even bursts) as a result of species diffusing in and out of the observation volume, as well as of any photodynamics taking place while a molecule traverses the observation volume.³⁶ FCS can distinguish between the fluorescence of free fluorophores and fluorophores bound to large objects. FCS provides a challenging test for the identity and integrity of self-assembled fluorescent nano-objects, but is still rarely used for studying such objects.

In addition to FCS, other methods based on the analysis of the fluctuations of the fluorescence from a small volume over time have been developed recently, in part as a result of progress in detection

electronics and computational speed. Of particular interest for the study of the interaction of fluorophores with larger objects is the PAID method³⁷ (Photon Arrival-time Interval Distribution) which extends upon FCS by including information on the brightness of the fluorescence species in the analysis, at the expense of a large computational effort for fitting models to the data. Contrary to PAID, FCS does not allow for the simultaneous determination of accurate values for the brightness, concentration and diffusion time of all species from a single experiment in the case where the brightness and occupancy for each species are initially unknown. Nevertheless, PAID is a computationally very demanding method, and FCS alone is appropriate for the study of the stability of fluorescent nano-objects.

Results and discussion

Predictions from quantum chemistry

The effect of noble metal particles on the radiative and nonradiative transitions of nearby fluorophores can be modeled quantum chemically in the framework of the polarizable continuum model (PCM).³³ The metal particle is described globally through its (complex) dielectric function which fully accounts for its optical properties. This approach has recently been applied to systems consisting of organic fluorophores and gold and silver nanoparticles.³⁸ It was found that these nanoparticles are very efficient excitation energy acceptors,^{39,40} in line with experimental observations of strong fluorescence quenching^{41–43} in fluorophore-functionalised noble metal nanoparticles.

In this section we investigate the effect of gold particles on the perylene monoimide (PMI) chromophore as a function of particle size, and chromophore distance and orientation. In particular two limit orientations have been explored, one with the PMI long axis longitudinally oriented to the spherical radius and one perpendicularly oriented (Fig. 2).

The PCM method gives access to the radiative rates of the PMI dye, which we will denote here by k_r . The fluorescence quantum yield of a single species, in the absence of a proximate metal sphere is given by eqn (1).

$$\Phi_F^0 = \frac{k_r^0}{k_r^0 + k_{nr}^0} \quad (1)$$

In the presence of a metal the fluorescence quantum yield (QY) becomes eqn (2).

$$\Phi_F^{\text{met}} = \frac{k_r^{\text{met}}}{k_r^{\text{met}} + k_{nr}^{\text{met}} + k_{nr}^0} \quad (2)$$

The presence of the metal introduces a change in the radiative rate k_r , and adds an extra term to the nonradiative decay. This term k_{nr}^{met} corresponds to excitation energy transfer (EET) from the chromophore to the metal particle. Here we will set k_{nr}^0 to zero for simplicity. This simplifying assumption will only induce a slight deviation because PMI and its derivatives have quantum yields (*vide infra*) that are sufficiently high for this assumption to work.

We also specify the relative brightness (RB, eqn (3)) of a fluorophore near a particle compared to the free fluorophore. RB represents fluorescence intensity at constant excitation power, as this is the quantity measured in fluorescence experiments.

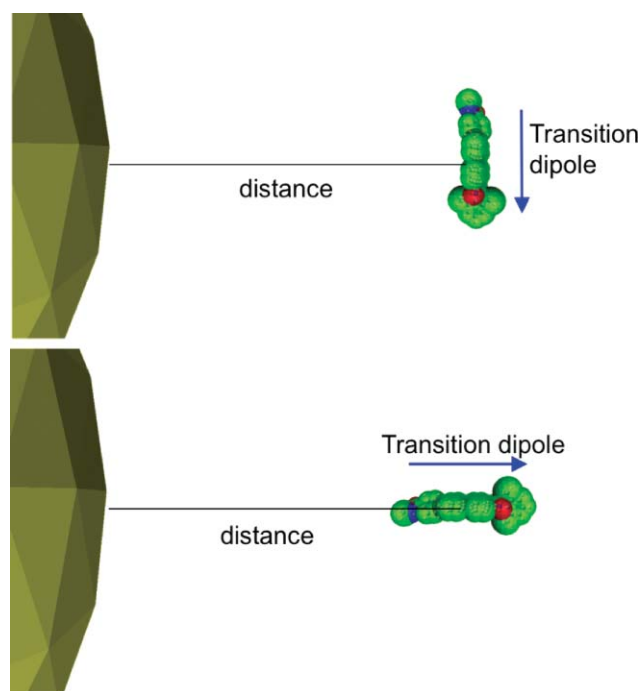


Fig. 2 Schematic of chromophore-particle setup, defining distance d (from the gold particle surface to the chromophore center of mass) and indicating perpendicular (top) and longitudinal (bottom) orientations. The chromophore is shown inside the proper PCM cavity.

$$RB = \frac{\epsilon^{\text{met}} \Phi_F^{\text{met}}}{\epsilon^0 \Phi_F^0} \text{ or } RB = \frac{\epsilon^{\text{met}}}{\epsilon^0} \Phi_F^{\text{met}} \text{ for } k_{\text{nr}}^0 = 0 \quad (3)$$

RB, contrary to the QY, also takes into account the effect of the metal particle on the absorption coefficient ϵ (the ratio $\epsilon^{\text{met}}/\epsilon^0$ accounts for the different population in the excited state induced by metal effects on the molecular absorption).

Within the same PCM approach we can couple the effect of the metal particle with that of a solvent (here DMF); solvent effects are also treated through the PCM description. All the details of the model can be found in references 33–35 and 38, whereas in the Materials and methods section we recall some aspects which are useful for the following analysis of the results.

Fig. 3 shows both the calculated quantum yield and the relative brightness for PMI at different distances from gold spherical particles of increasing size (diameter = 34 Å, 120 Å and 250 Å, respectively) in DMF. The two different limit orientations described in Fig. 2 have been studied, but here, for the sake of simplicity we only report the longitudinal case (the perpendicular results are reported in the ESI, Fig. SI-1‡).

By comparing the two graphs (QY and RB) we can dissect the effect of the gold particle on the excitation and de-excitation process. The effects of distance from the metal particle on k_r (and ϵ) and k_{nr} have qualitatively similar trends for all three rates: the shorter the distances, the higher the rate. However, in the region of short distances, k_{nr} is much greater than k_r , so it determines the quenching of the fluorescence. Even the enhancement of ϵ at short distances is not able to offset the large quenching of fluorescence, resulting in an RB value smaller than 1. For long distances and small particles, QY and RB thus trivially approach the free-

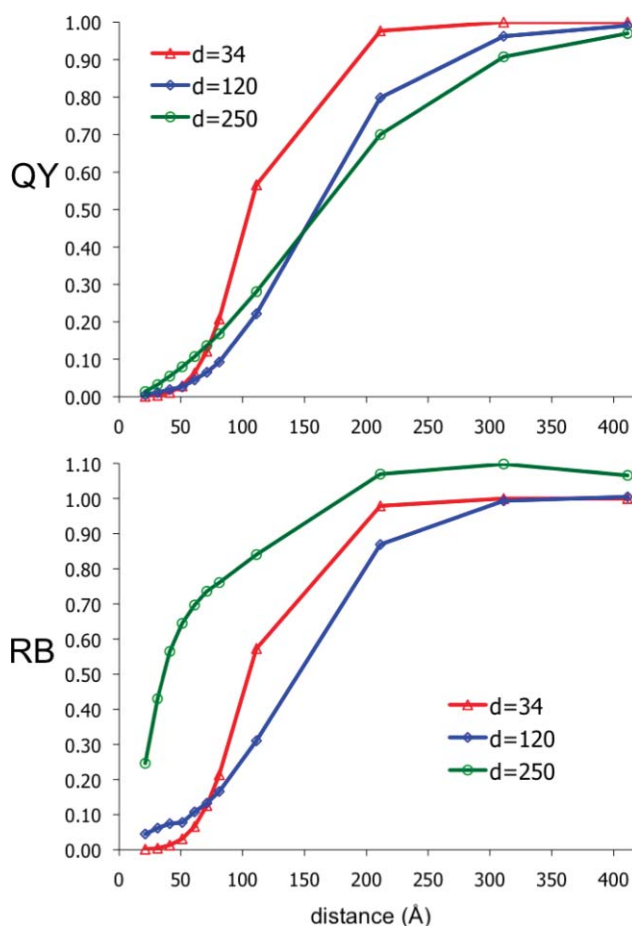


Fig. 3 Theoretical fluorescence quantum yield and relative brightness as a function of distance of the PMI chromophore near a gold nanoparticle in DMF at longitudinal orientation, for different gold particle diameters ($d = 34$, 120 and 250 Å). Top: quantum yield (Φ_F vs. distance), bottom: relative brightness (RB vs. distance).

molecule limit, *i.e.*, 1. However, a marked difference between QY and RB can be seen for the largest particle ($d = 250$ Å). The effects of the radius of the metal particle for small metal-molecule distances differ for k_r and k_{nr} (see ESI, Fig. SI-2‡). Larger radii maximize k_r (and ϵ)^{44–46} but are relatively less effective in increasing k_{nr} . This means that it is possible to increase k_r (and ϵ) more than k_{nr} and to produce an increase in RB to values larger than 1. This is indeed observed in Fig. 3 for 200–300 Å distances for which an overall enhancement of the fluorescence intensity is predicted. We finally note, that this net enhancement is not present in the case of perpendicular orientation (ESI, Fig. SI-1‡) as in that case there is not the co-linearity between the chromophore transition moment (directed along the main axis) and the sphere-induced dipole which is present in the longitudinal orientation and which gives rise to a large increase in both k_r and ϵ .

The distance between the PMI chromophore and the gold nanoparticle in assemblies of PMImSS will be around 31 Å at most (estimated using a fully extended chain in a MM2 force field model), and the fluorescence in the systems studied here is thus expected to be strongly quenched. The interaction of PMImSS ligands with gold nanoparticles will be accompanied by a strongly decreasing fluorescence.

Table 1 Photophysical data for PMIester and PMImSS in different solvent systems: wavelengths of maximum absorption (λ_{abs}) and emission (λ_{em}), fluorescence quantum yields (Φ_F) and decay times (τ_F), and molar absorption coefficients (ϵ)

	Solvent	$\lambda_{\text{abs}}/\text{nm}$	$\lambda_{\text{em}}/\text{nm}$	Φ_F	τ_F/ns	$\epsilon/\text{M}^{-1}\text{cm}^{-1}$
PMImSS	DMF	513	580	0.72	3.9	
	DMF–H ₂ O (8 : 2 v/v)	517	588	0.63	3.7	
	toluene	489 ^a , 517 ^a	548 ^a , 589 ^a	0.80	3.8	
PMIester	DMF	514	575	0.72		3.7×10^4
	DMF–H ₂ O (8 : 2 v/v)	516	589	0.62		3.3×10^4
	toluene	489 ^a , 517 ^a	544 ^a , 585 ^a	0.80	3.9	3.2×10^4

^a Vibronically structured band.

Photophysical properties of the perylene-monoimide chromophore and the functional ligand

Before addressing the interaction of PMImSS with gold nanoparticles we describe the photophysical properties of the PMIester model chromophore, and the free PMImSS ligand in solution. Comparison of the absorption and emission spectra of PMIester and PMImSS (Fig. 4), and their fluorescence quantum yields and lifetimes (Table 1) shows that the introduction of the disulfide tail does not significantly alter the photophysics of the PMI chromophore. Because of the small quantity of PMImSS ligand available, its molar absorption coefficient was not measured. In view of the similarity of the chromophores, and because of the similar fluorescence quantum yields and lifetimes of PMIester and PMImSS, the molar absorption coefficients of both compounds are very likely to be comparable, and therefore the extinction coefficients of PMIester will also be used for PMImSS.

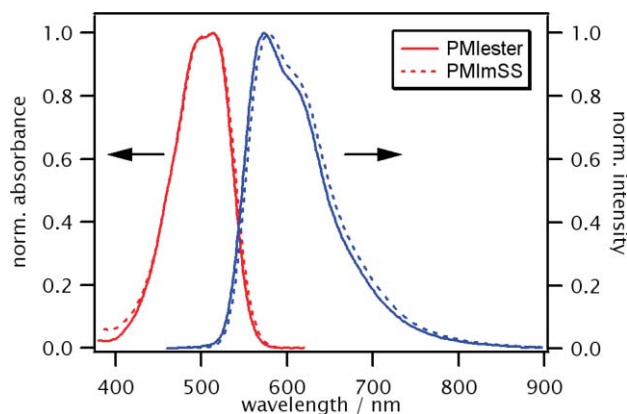


Fig. 4 Absorption and corrected emission ($\lambda_{\text{exc}} = 470 \text{ nm}$) spectra of PMIester (solid line) and PMImSS (dashed line) in air-equilibrated DMF.

The fluorescence quantum yields reported here are lower than quantum yields for similar PMI-based compounds reported in the literature, e.g. derivatives of PMI used to build dendrimeric architectures were found to have a fluorescence quantum yield of 0.98 in toluene.⁴⁷ The free acid obtained after hydrolysis of PMIester has been reported to have a fluorescence quantum yield of 0.93 in toluene.²⁹ In that work, Rhodamine 101 in methanol and Cresyl Violet in ethanol were used as fluorescence standards, whereas we used Perylene Orange in toluene ($\Phi_F = 0.99$),⁴⁸ because

of its good spectral overlap with PMIester and PMImSS. It may thus be that the use of different reference compounds leads to the discrepancy between the values in this work and those found elsewhere. Langhals *et al.* have determined the FQY of an unsubstituted PMI derivatives using Perylene Orange as the standard^{49,50} and found values between 0.84 and 0.88.

The fluorescence decay of the free PMImSS ligand, like that of the PMIester compound, is monoexponential, indicating the presence of only one single emitting species. This is an improvement compared to FITC derivatives used in our previous work.^{17,51} Even simple FITC adducts display multiexponential decay and fluorescent behaviour involving several emitting species²³ This complicates the assessment of the effect of nearby metal particles on the photophysics in FITC-functionalised assemblies. The PMImSS ligand studied here is better suited for such studies.

PMImSS is not soluble in water, but well soluble in DMF and toluene. For studying interactions with AuNPs it is desirable that at least a small amount of water does not induce aggregation or a complete loss of fluorescence. Fig. 5 shows that small fractions of water in DMF (up to 20 vol%, which corresponds to a molar fraction of 50%) do not strongly quench the fluorescence. The fluorescence decay remains mono-exponential with a fluorescence decay time τ_F close (3.7 ns) to the decay in pure DMF (3.9 ns). This demonstrates that small amounts of water can be used in our experiments without sacrificing the favorable photophysics of PMImSS. However, at higher concentrations (beyond 30 vol%) of water in DMF, aggregation of PMImSS does occur, leading to multiexponential fluorescence decays, and reduced overall fluorescence quantum yields.

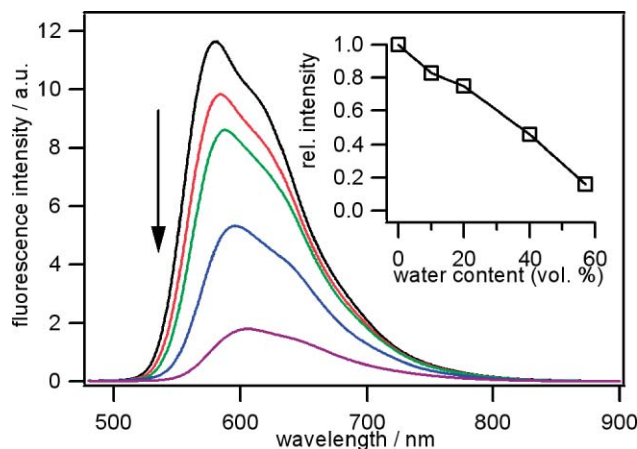


Fig. 5 Evolution of emission spectrum and fluorescence intensity (inset) of PMImSS in DMF–water mixtures as a function of DMF–water ratio (LED excitation at 458 nm, uncorrected spectra, CCD detector). Concentration of PMImSS was kept constant at 1 μM .

Based on the fluorescence quantum yield and observed fluorescence decay time, the radiative lifetime (or ‘natural lifetime’, $\tau_R = \tau_F / \Phi_F$) of PMImSS increases from 5.4 ns in pure DMF to 5.9 ns in DMF–water 8 : 2 (v/v). This decrease in radiative transition probability is in line with the slight decrease in molar absorption coefficient experienced by the PMIester chromophore on going from DMF to DMF–water ($3.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ in DMF, $3.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ in DMF–water 8 : 2 (v/v)). Part of the fluorescence quenching of PMImSS when adding water of DMF thus comes

Table 2 Diffusion times determined by fluorescence correlation spectroscopy for PMImSS in DMF and DMF–H₂O

Solvent	τ_D /ms
DMF	0.12 ($\pm$ 0.01)
DMF–H ₂ O (8 : 2 v/v)	0.16 ($\pm$ 0.02)

from a reduced radiative rate, giving nonradiative processes more importance. Another part of the fluorescence quenching is due to enhanced nonradiative processes, which may be the result of hydrogen bonding between the added water and the PMI chromophore.

One additional issue with the PMImSS ligand is to obtain solutions in which all molecules exist as isolated, individual entities, without any aggregates present. Previous studies⁵¹ on fluorescein-labeled disulfide ligands in water were made complicated by the existence of aggregates. These aggregates were identified using fluorescence correlation spectroscopy (FCS) as big, slowly diffusing species, which can easily be mistaken for functionalised nanoparticles. Solutions containing PMImSS alone should therefore not contain any slowly diffusing aggregates.

In this work we have used FCS to study the solutions of PMImSS in DMF and DMF–water (80 : 20 by volume). The laser power, which is on the order of 3.5 kW cm^{−2} on the sample, is far below the saturation limit in order to avoid contribution from blinking^{26,27} resulting from singlet-to-triplet intersystem crossing. We have chosen to remove the confocal pinhole to favor a high signal level. The size of the observation volume was thus determined by the core diameter of the collection fiber, which takes on the role as the confocal pinhole. From the measured FCS curve (see ESI, Fig. SI-3[†]), the experimental diffusion time τ_D is obtained by fitting a single-species model (see Materials and methods). This diffusion time τ_D corresponds to the average amount of time the species spends in the observation volume.

The values of the diffusion times of PMImSS that we have measured are reported in Table 2. By taking into account the measured diffusion time of Rh6G molecules in water and the viscosity of DMF and DMF–water mixtures,⁵² the hydrodynamic radius of PMImSS in DMF and in the mixture of DMF–water (which has higher viscosity than either pure water or pure DMF) can be estimated to be approximately 2.5 to 3 times larger than that of Rh6G in water (5.9 Å⁵³), in line with the radius expected for unaggregated PMImSS molecules (15 Å). The effect of the change in refractive index on the focal volume has been ignored at present.

Fluorescence correlation spectroscopy on solutions of PMImSS in DMF and DMF–water (80 : 20 by volume) confirms the absence of aggregates, already indicated by the monoexponential fluorescence decay (τ_F = 3.7 ns), and the fluorescence quantum yield which does not radically decrease on going from pure DMF to DMF–water (8 : 2 by volume).

Stability of citrate-stabilized gold nanoparticles in water-organic solvent systems

As mentioned previously, PMImSS is not soluble in pure water or in solvent mixtures with high water content. In order to study its interaction with gold nanoparticles, it is necessary to identify solvent systems that efficiently dissolve the organic dye, while still yielding stable suspensions of the excess-citrate stabilised gold

nanoparticles (xCS-AuNPs). For instance, a 50 : 50 (by volume) mixture of THF and water has been used to functionalise water-borne 13 nm xCS-AuNPs with hydrophobic calixarene ligands.⁵⁴

In order to identify suitable mixtures, we have studied the colloidal stability of xCS-AuNP in several water–THF and water–DMF mixtures. The plasmon resonance absorption band is an excellent probe for even slight aggregation of gold nanoparticles, because of its high sensitivity toward the dielectric environment of the metal nanoparticles. Upon aggregation of the particles, an intense long-wavelength band (>600 nm) will develop, stemming from particle dimers and increasingly larger aggregates. It is the development of this absorption band that leads to the well-known colour change from red to blue when unprotected gold nanoparticles are exposed to agglomerating agents such as NaCl. The long-wavelength band is readily detected using UV/Vis absorption spectroscopy.

The spectra in Fig. 6 demonstrate that xCS-AuNPs are very stable in DMF–water solvents, since no additional aggregation-related absorption band develops in these solvent systems. At high DMF concentrations the plasmon resonance sharpens up, which is probably a result of interactions of the DMF molecules with the particle surface. No additional absorption bands are detected, whereas in water–THF long-wavelength bands appear (shoulder at 650 nm), indicative of aggregation. Interestingly, the additional absorption band found for water–THF does not evolve significantly in the course of several days. This indicates the formation of stable aggregates of only a limited number of particles. The formation of a shoulder near 650 nm is indicative of

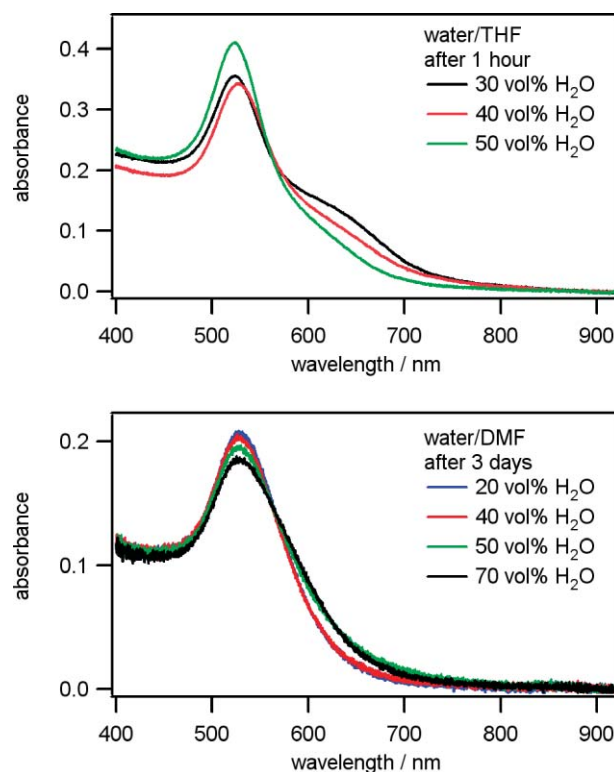


Fig. 6 Absorption spectra of xCS-AuNP in water–THF mixtures (after 1 h, top), and in water–DMF mixtures (after 3 days, bottom). xCS-AuNP concentrations were held at 1.7 nM (THF–water) and 0.8 nM (DMF–water).

the formation of dimers or bent trimers.^{55–57} Larger assemblies absorb at longer wavelengths. A mixture of water–THF thus seems to induce and stabilise the formation of particle aggregates consisting of only a few particles. Such a phenomenon may possibly be used to conveniently control the construction of multi-particle assemblies.

Interaction between PMImSS and gold nanoparticles

Because of the enhanced stability of xCS-AuNP in DMF–water mixtures, compared to THF–water, the studies involving the interaction between PMImSS and xCS-AuNP were carried out in a DMF–water (8 : 2 by volume) mixture. Solutions of PMImSS in DMF–water (8 : 2) were prepared, keeping the concentration of PMImSS constant, but gradually increasing the concentration of xCS-AuNPs. The fluorescence emission spectra of these samples are shown in Fig. 7, together with the corresponding titration curve. The concentration of gold nanoparticles is expressed as the estimated number of thiolate binding sites (see Materials and methods), *i.e.* the concentration of thiol or disulfide sulfur atoms that can cover the surface of these particles.

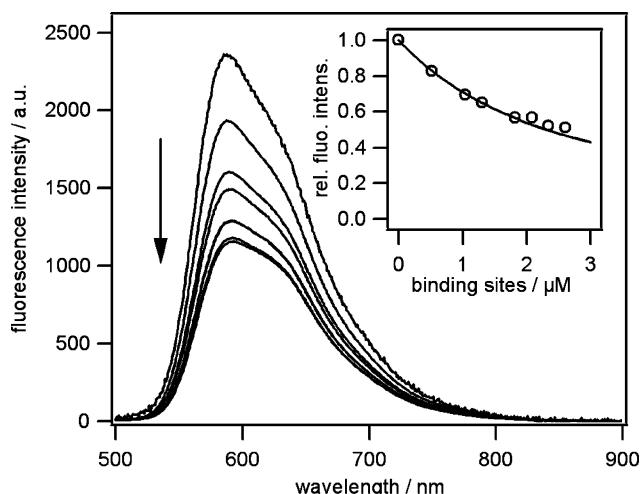


Fig. 7 Fluorescence titration of PMImSS with xCS-AuNP in DMF–water (8 : 2 by volume). PMImSS concentration was held constant at 0.3 μM (corresponding to 0.6 μM of ligating sulfur atoms). The number of particle binding sites was calculated from the xCS-AuNP absorption spectrum (see Materials and methods). Main panel: uncorrected fluorescence spectra for increasing xCS-AuNP concentration (LED excitation at 458 nm). Inset: normalized integrated fluorescence intensity as a function of binding site concentration. The solid line is the theoretical curve for a simple binary equilibrium between fully fluorescent free chromophores and completely quenched bound chromophores, using the experimental concentrations and an association constant of $5.0 \times 10^5 \text{ M}^{-1}$.

Contrary to what would be expected given the reported high affinity of thiols and disulfides towards gold surfaces,¹⁴ the titration curve falls off slowly, instead of decreasing linearly with increasing AuNP particle concentration until the equivalence point is reached. The titration does not end after 1 equivalent of particle binding sites (which would be at 0.6 μM), but many more particles are needed to bind even half of all PMImSS present. A large fraction of unbound, fluorescent PMImSS is always present. The titration curve is actually described well using a dynamic equilibrium involving two species (PMImSS and a AuNP binding

site) forming a single nonfluorescent complex (particle-bound PMImSS), with an association constant of $5.0 \times 10^5 \text{ M}^{-1}$. Reversing the titration, adding PMImSS to a solution of xCS-AuNP, also gives titration curves compatible with this model. According to the binary dynamic equilibrium model that yields the fit in the inset of Fig. 7, the observed remaining fluorescence of PMImSS in presence of AuNPs stems virtually exclusively from unbound PMImSS molecules.

We investigated if similar behaviour occurred for AuNPs of a different type, and in different solvents (Fig. 8). For this experiment, AuNPs stabilised by tetra(n-octyl)ammonium bromide (TOAB-AuNPs) were used. These particles are soluble in toluene and in DMF. Similar binding behaviour is observed for all three systems of PMImSS with AuNPs, indicating that the surprisingly low affinity between the PMImSS disulfide and gold nanoparticles is not limited to xCS-AuNPs.

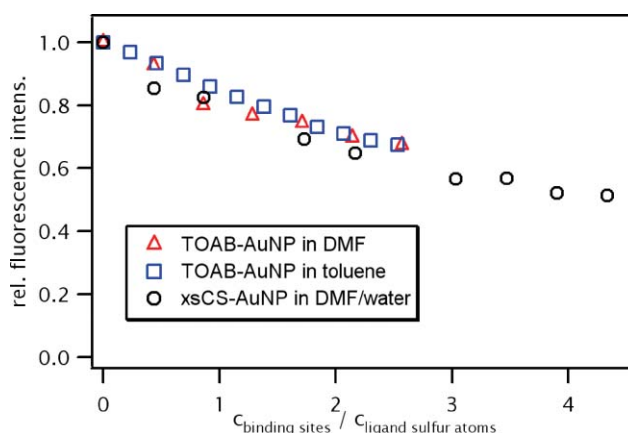


Fig. 8 Fluorescence titration (integrated intensity, LED excitation at 458 nm) of PMImSS with two different AuNPs type in different solvent systems: xCS-AuNP in DMF–water (8 : 2 by volume), TOAB-AuNP in DMF and toluene. PMImSS concentration was held constant, and AuNP particle concentration was varied.

The existence of a binary equilibrium between free and bound PMImSS suggests that in order to bind a significant fraction of ligands, higher concentrations of both ligands and particles are necessary. It also suggests that significant desorption will take place when solutions of functionalised particles are diluted, which is the case in fluorescence experiments, in particular in fluorescence correlation spectroscopy.

Perylene-monoimide functionalised particles

In order to study potential ligand desorption from functionalised particles, gold nanoparticles coated completely with PMImSS ligands were made by mixing an excess of PMImSS in DMF and xCS-AuNP in water to give a mixture DMF–water 8 : 2 by volume. The excess of ligands corresponds to 4 times the ligands needed to cover all of the theoretically available binding sites on the particles,¹⁷ *i.e.* ~ 2800 binding sites per particle for xCS-AuNP particles. This theoretical number corresponds to a dense self-assembled monolayer of thiols on gold, and is in line with observations by Hostetler *et al.*⁵⁸ Particle concentrations were determined by UV/Vis spectroscopy using known extinction coefficients for gold nanoparticles (see Materials and methods).

The same extinction coefficient and number of binding sites were used to obtain association constant for the data in Fig. 7.

The resulting suspension of functionalised particles with excess free ligands (12 μM in PMImSS, 3 μM in AuNP binding sites, DMF–water 8 : 2) was first concentrated by centrifugation (14 000 rpm, 24 000 g, 10 min), after which the supernatant was removed, thus removing a significant part of the excess of PMImSS, which remains in solution and does not concentrate in the pellet, contrary to the functionalised particles. Fresh DMF was added to the pellet and the new suspension was centrifuged again, and the supernatant replaced by fresh DMF again, in order to wash away the excess of ligands. The red coloration of the particles concentrated in the pellet was readily diluted into the added DMF, which indicates successful resuspension of the particles. After each centrifugation cycle, the fluorescence of the supernatant was monitored, in order to check efficient removal of free PMImSS from the suspension.

Functionalisation of the AuNPs by PMImSS was evident from a control experiment in which PMIester instead of PMImSS was used. Already after one centrifugation cycle, the pellet was not red anymore, but black, and resuspension of the pellet in DMF was not achieved, indicating the necessity of the presence of the disulfide motif in PMImSS to stabilise the particles.

The freshly purified suspensions were immediately studied by FCS, in order to identify the presence of free and bound ligands. The initial FCS curve demonstrates the exclusive presence of large diffusing fluorescent objects ($\tau_{\text{diff}} = 3800 \mu\text{s}$) combined with a short exponential decay component ($\tau = 28 \mu\text{s}$), compatible with a blinking process. At zero time lag ($\tau = 0$), the contribution of a blinking process between a dark and a bright state is given by a factor of $(1 + p)$, in which p is the ratio between the species in the dark state and the species in the bright state. In the present case, p has a value of 0.1, which indicates that the objects are in their bright state during 90% of the time spent in the focal volume. Different processes can be at the origin of the blinking process such as triplet blinking or ligand desorption for example. Even if the triplet blinking does not appear on the FCS curve of the pure PMImSS solution at the same excitation power (see ESI, Fig. SI-3†), the dynamics of the singlet-to-triplet intercrossing process can be modified by the presence of numerous close chromophores linked to the particle, as has been demonstrated in the case of a multichromophoric dendrimer, decorated with peryleneimides.²⁸ The dynamics of the energy transfer between close chromophores is indeed complex, since it has recently been demonstrated that in the case of a multichromophoric gold core system the energy transfer between close chromophores can be inhibited.²⁰ The ligand adsorption-desorption events can also produce a blinking signature since it influences the energy transfer between the chromophores.

There is no signature of any free ligands, as demonstrated by the absence of a continuous background in the burst histogram (Fig. 9, top), which contains only strong fluorescence bursts. The fluorescent objects display bursts that are roughly 10 times as intense as the bursts from single PMImSS molecules. If the number of fluorophores per AuNP is estimated to be 200 (based on the FCS amplitudes, *vide infra*), the relative brightness of an individual particle-bound fluorophore is found to be roughly 5% of that of a free ligand. This is not incompatible with the theoretical prediction in Fig. 3, taking a PMImSS–AuNP distance of 31 Å (fully extended alkyl-tetraethyleneglycol spacer) and a 12 nm (120 Å) diameter

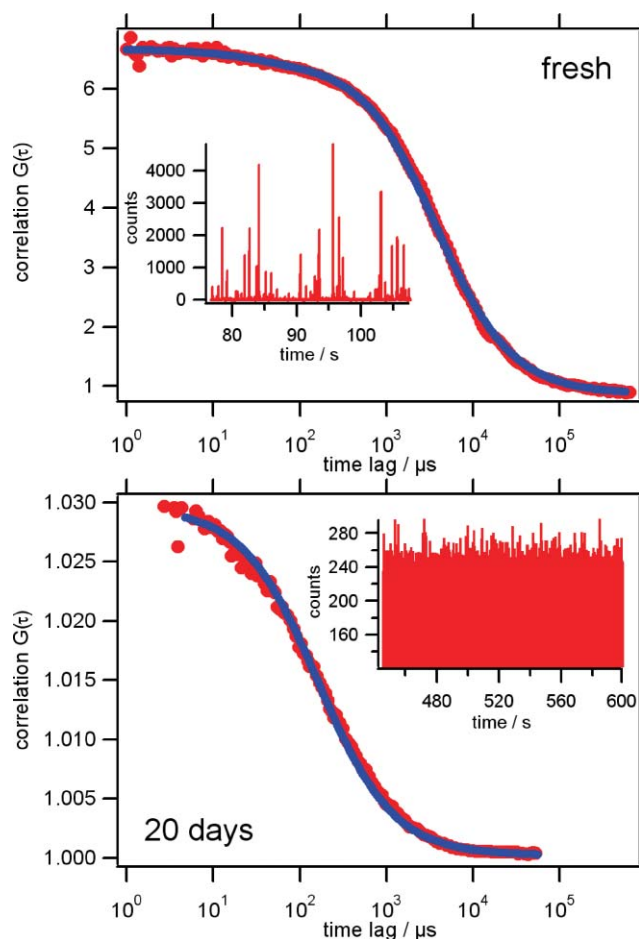


Fig. 9 Fluorescence correlation traces of freshly purified PMImSS–AuNP in DMF (top) and of the same suspension 20 days after purification (bottom). The insets consist of time traces of the fluorescence count rate (counts per 10 ms), demonstrating the sparse, intense bursts from large objects in the fresh sample, and continuously fluctuating fluorescence from the free chromophores in the aged sample. The bin time of the time traces is 10 ms.

gold nanoparticle, which has a predicted relative brightness of 0.06.

After 20 days, the FCS trace of the sample has evolved: the signal is now dominated by the fluorescence from rapidly diffusing fluorophores as shown on the correlation curve (Fig. 9, bottom). This means that the PMImSS–AuNP have liberated a large fraction of their capping ligands. Because of the extremely low concentrations (approx. pM in particles) used in fluorescence correlation spectroscopy, we are not able to determine the fate of the gold particles. No precipitate is found at the bottom of the container. At higher concentrations (approx. nM in particles) the solutions of PMImSS–AuNP are stable over several weeks, and the absorption shows no degradation of the surface plasmon band. In spite of the loss of ligands, the particles are likely to be stable in DMF at low concentrations, based on experiments in which small quantities of an aqueous xCS–AuNP suspension are added to DMF.

The correlation amplitude of the FCS trace has considerably decreased compared to the fresh sample. Since this correlation amplitude is inversely proportional to the average number of

diffusing species, the number of emitting objects has greatly increased. The increase can be estimated to be a factor of 200, indicating significant ligand desorption after 20 days. This number is lower than the theoretical estimation of the number of fluorophores on a fully functionalised PMImSS-AuNP, which gives 1400 chromophores per particle (based on the previously mentioned 2800 available thiol binding sites). As mentioned above, the theoretical estimate is based on a dense monolayer of thiols. It may be that the presence of both the PMI chromophore and the oligoethyleneglycol chain introduces disorder in the monolayer leading to less available room for ligands.

In a separate experiment, we investigated a one-day old sample of PMImSS-AuNP in DMF. One day refers to the time that the sample has existed since it was purified by centrifugation. This sample contains both functionalised particles and free fluorophores. We used the combination of time-correlated single photon counting and fluorescence correlation spectroscopy offered by the acquisition electronics on the FCS experiment. This allows us to extract fluorescence lifetime data from the same measurements. Interestingly, the fluorescence decay is no longer monoexponential. After deconvolution of the instrument response, several contributions to the fluorescence decay are found. Beyond the contribution of the free ligands ($\tau = 3.79$ ns on the equipment used for FCS), the fit indicates the presence of two components with short decay times. One has a characteristic time of the same order or shorter than that of the impulse response (≈ 50 ps) and the other, weaker, has a decay time of about 320 ps. These short decays correspond to species whose fluorescence is quenched.

The calculation of the correlation function of the quenched species alone can be obtained by numerical post-acquisition temporal filtering. The fit of the corresponding correlation curve demonstrates that the quickly diffusing free ligands do not contribute to the short fluorescence decay component, and that this “quenched” signal arises from the large objects (see ESI, Fig. SI-4†). The correlation curve additionally displays a 50 μ s short exponentially decaying component, the signature of a blinking process, potentially related to the ligand desorption process, or to triplet blinking.

Concluding remarks

The affinity of the PMImSS disulfide ligand for gold nanoparticles is not as large as usually assumed for thiols and disulfides on gold. Fully functionalised PMImSS-AuNP particles lose their ligands in dilute solution, as demonstrated by the fluorescence correlation spectroscopy. Fluorimetric titration curves of PMImSS with AuNPs do not show the typical shape for high affinity complexation (*i.e.* a linear decrease before equivalence, followed by a flat line after equivalence). Our experimental results rather suggest the existence of an equilibrium between free and bound fluorophores with an apparent affinity constant of 5×10^{-5} M. A suitable fit to the data is obtained with a model involving a brightly fluorescent free ligand and a strongly quenched bound ligand, implying that the fluorescence observed in the titration experiments mainly comes from unbound ligands. From the fluorescence correlation results, it is estimated that the brightness of a single particle-bound fluorophore is at most 5% of the brightness of a free fluorophore, in line with the theoretical predictions.

The nature of the species involved in the equilibrium is difficult to determine, but some hypotheses can be made if recent literature is taken into account. Recent literature suggests that adsorbed thiols and disulfides may desorb from gold particles in the form of gold(I) thiolates.^{59,60} One possibility may thus be that in our system the incoming PMImSS disulfide is cleaved into its geminate thiolates on the surface of the gold particles, which then desorb in the form of Au(I) thiolates (Fig. 10). Subsequently, these gold thiolates can then re-adsorb, effectively setting up the dynamic equilibrium giving rise to the titration curves found in the present work.

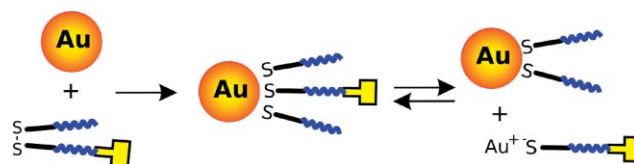


Fig. 10 Hypothesis concerning disulfide adsorption onto gold particles, and subsequent desorption/adsorption of gold(I) thiolates.

Further work is planned to elucidate the nature of the species involved, in particular the analysis of the supernatant obtained from the centrifugation of an aged sample of purified (free fluorophore-depleted) PMImSS-AuNP. Electrospray mass spectrometry, potentially coupled to liquid chromatography, may be able to give a reliable indication of the chemical species that desorb from functionalised gold nanoparticles over time.

The hypothesis outlined in Fig. 10 is corroborated by a re-interpretation of results obtained on symmetrical bis-pyrenyl disulfide ligands published in this journal.⁶¹ The excimer emission of these ligands disappears in the presence of gold nanoparticles. The original interpretation was that the disappearance of the excimers and their emission was the result of cleavage of the disulfide bond and migration of the geminate thiolates over the surface the nanoparticles. Such a migration of thiolate ligands has been observed for flat gold surfaces in high vacuum,^{62–64} but recent studies using electron spin resonance labels suggest^{59,65} that such a migration process should be much slower for nanoparticles in solution than the dynamics observed by us.

A different interpretation of the published experimental results, which would be in agreement with the interpretation of the results described here, would be the cleavage of the disulfide followed by desorption and re-adsorption of the geminate thiolates. The cleavage of the symmetrical bis-pyrenyl disulfide would explain the disappearance of the excimer emission. Such a desorption–re-adsorption mechanism has also been proposed recently by Ionita *et al.* on basis on their electron paramagnetic resonance results using spin-labeled ligands.⁶⁵

Our findings suggest that systematic photophysical studies of thiol and disulfide functionalised particles should take into account the potential presence of desorbed ligands. The limited apparent affinity of disulfides for gold nanoparticles, and their tendency to desorb at low concentrations, suggests that stronger, “multidentate” ligands⁶⁶ are better suited for the construction of hybrid organic-inorganic nano-objects, when robustness is a requirement, such as in medical diagnostics or biological imaging. Furthermore, longer spacers, extending beyond the reach of small ligands, would be helpful in further studying the energy transfer

from organic dyes to metal nanoparticles, as well as the inclusion of noble metal particles of different sizes and composition.

Materials and methods

Computational methods

Within the PCM framework, the interaction between the molecule, the solvent and the metal is electrostatic in nature, and it is due to their mutual polarization. In particular, the molecule is described quantum-mechanically using an effective Hamiltonian obtained by adding the *in vacuo* electronic Hamiltonian and the electrostatic interaction energy between the molecular charge density (nuclei + electrons) and the polarization of the solvent and of the metal induced by the molecular charge density itself. Such a polarization is conveniently expressed in terms of apparent charges placed on the metal surface and on the surface of the molecular cavity hosting the chromophore as a solute. The electronic excitation energies and transition densities of the molecule are calculated within the time-dependent density functional theory (TD-DFT) approach properly modified so to include a term that represents the interaction of the transition density with the oscillating metal and solvent polarization induced by the transition density itself. This interaction is again described with apparent surface charges determined in terms of the frequency-dependent dielectric permittivity of the metal and the solvent (the frequency is chosen to be equal to that of the studied molecular electronic transition).

To describe the optical response of the gold particles the frequency-dependent dielectric permittivity was taken from experimental data⁶⁷ and corrected for the limited mean free-path according to the Drude model,⁶⁸ following eqn (4).

$$\epsilon^{\text{met}}(\omega, R) = \epsilon_{\text{exp}}^{\text{met}}(\omega) + \Omega^2 \left\{ \frac{1}{\omega \left(\omega + \frac{i}{\tau} \right)} - \frac{1}{\omega \left(\omega + i \frac{(R + v_F \tau)}{\tau R} \right)} \right\} \quad (4)$$

In this expression R is the effective radius of the metal nanoparticle, Ω is the plasma frequency (0.293 a.u.), v_F is the Fermi velocity (0.64 a.u.), and τ is the bulk relaxation time (306 a.u.). For the bulk permittivity the following values have been used: $\text{Re}\{\epsilon^{\text{met}}\} = -6.290$, $\text{Im}\{\epsilon^{\text{met}}\} = 2.0422$, and $(\text{Re}\{\epsilon^{\text{met}}\} = -8.944, \text{Im}\{\epsilon^{\text{met}}\} = 1.416$ for the absorption and the emission process, respectively. All the values used are taken from ref. 67.

For the chromophore the (TD)DFT calculations (including geometry optimization of both ground and excited state) have been performed using Becke three parameters hybrid functional with Lee–Yang–Parr correlation function (B3LYP)^{69,70} and the basis set chosen was 6-31+G(d,p).

To include solvent effects, the chromophore was enclosed in a cavity made up of a combination of spheres centered on C, O, N, and CH₃ with radii 1.925, 1.830, 1.750, and 2.525 Å, respectively. The solvent (DMF) was represented by its static dielectric constant ($\epsilon = 37.22$), and by its optical dielectric constant, ($\epsilon_{\infty} = 2.18$).

All the calculations have been performed using a locally modified version of the Gaussian package⁷¹ in which we have implemented the described PCM-metal approach.

Synthesis of PMI-ester and PMI-mSS

Synthesis of PMI-ester (*N*-(2,6-diisopropylphenyl)-9-(4-ethyl-carboxyphenyl)-perylene-3,4-dicarboximide). Compounds *N*-(2,6-diisopropylphenyl)-9-bromoperylene-3,4-dicarboximide⁷² (0.170 g, 0.30 mmol) and ethyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoate (0.135 g, 0.49 mmol, 1.6 equiv.) were put in a flask and put under a nitrogen atmosphere. Then nitrogen-purged toluene (50 mL) was added. Thereafter Pd(OAc)₂ (10.1 mg, 45 μmol, 15 mol%) and triphenylphosphine (49.5 mg, 0.19 mmol, 4.2 equiv. compared to Pd-catalyst) were added. Finally, a solution of K₂CO₃ in water (2 M, 15 mL), which had been purged with nitrogen, was added and the reaction mixture was heated to 90 °C and stirred overnight under nitrogen atmosphere. After cooling to room temperature the layers were separated and the organic phase was concentrated to dryness. The product was obtained (110 mg, 58%) after column chromatography of the product mixture over silica (eluent: CH₂Cl₂). ¹H NMR (400 MHz, CDCl₃): δ 8.66 (dd, 2H, $J = 8$ Hz, 1.6 Hz), 8.49 (m, 4H), 8.23 (d, 2H, $J = 8$ Hz), 7.93 (d, 1H, $J = 8$ Hz), 7.62 (m, 4H), 7.49 (t, 1H, $J = 8$ Hz), 7.34 (d, 2H, $J = 8$ Hz), 4.48 (q, 2H, $J = 7$ Hz), 2.78, (sept, 2H, $J = 7$ Hz), 1.46 (t, 3H, $J = 7$ Hz), 1.18 (d, 12H, $J = 7$ Hz). MS (FAB+) m/z (%) = 630.3 (100%, M–H⁺); (calcd. C₄₃H₃₅NO₄ = 629.26).

Synthesis of *N*-(2,6-diisopropylphenyl)-9-(4-carboxyphenyl)-perylene-3,4-dicarboximide. A solution of KOH in water (2 M, 0.5 mL) was added to a solution of PMIester (28 mg, 45 μmol) in freshly distilled THF (5 mL), after which the mixture was heated in an oil bath of 80 °C. The progress of the reaction was monitored by TLC and after refluxing for 24 h the TLC spot of PMIester had disappeared. The reaction mixture was poured into a solution of HCl in water (2 M, 20 mL). The precipitate was filtered and washed with water. A red solid was collected (26 mg, 43 μmol, 97%) and analysed with ¹H NMR and mass. ¹H NMR (400 MHz, CDCl₃): δ 8.68 (dd, 2H, $J = 8$ Hz, 2.4 Hz), 8.52 (m, 4H), 8.31 (d, 2H, $J = 8$ Hz), 7.95 (d, 1H, $J = 8$ Hz), 7.64 (m, 4H), 7.49 (t, 1H, $J = 8$ Hz), 7.34 (d, 2H, $J = 8$ Hz), 2.78, (sept, 2H, $J = 7$ Hz), 1.18 (d, 12H, $J = 7$ Hz). MS (FAB+) m/z (%) = 602.2 (100%, M–H⁺); (calcd. C₄₁H₃₁NO₄ = 601.23).

Synthesis of 3,6,9,12-tetraoxa-24,25-dithiatritriacontan-1-amine. The dissymmetric mono-amino terminated disulfide that forms the basis for PMImSS was synthesised by cleaving the sulfur bridge of the symmetric diamino-tetraethyleneglycolalkyldisulfide using a phosphine, followed by reoxidation by molecular oxygen in the presence of excess n-octanethiol. A solution of 3,6,9,12,37,40,43,46-octa-oxa-24,25-dithiaoctatetracontane-1,48-diamine¹⁷ (1.0 g, 1.32 mmol) in dry DMF (10 mL) with n-octanethiol (6 equiv.) and tri-(n-butyl)phosphine (0.9 equiv.) was stirred under argon for 12h. Subsequently the solution was exposed to air for 3 days while stirring. The solution was filtered to remove the tri-(n-butyl)phosphine oxide, and the filtrate was concentrated under vacuum and purified by column chromatography on silica gel with 1:1:0.3 ethyl acetate/heptane/Et₃N (R_f 0.2, ninhydrine) to obtain the 3,6,9,12-tetraoxa-24,25-dithiatritriacontan-1-amine in 63% yield (880 mg). ¹H NMR (300 MHz, MeOD): 3.7 (m, 14H), 3.49 (t, 2H), 2.83 (t, 2H), 2.70 (t, 4H), 1.34 (m, 30H), 0.85 (t, 3H).

Synthesis of *N*-(2,6-diisopropylphenyl)-9-(4-(*N*-3,6,9,12-tetraoxa-23,24-dithia-dotriacontylcarbonyl)phenyl)-perylene-3,4-dicarboximide (PMI-mSS). A solution of the perylene carboxylic acid derivative *N*-(2,6-diisopropylphenyl)-9-(4-carboxyphenyl)-perylene-3,4-dicarboximide (20 mg, 0.033 mmol) and benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP, 22 mg, 0.050 mmol, 1.5 equiv.) in DMF (5 mL) was stirred for 30 min at room temperature. A solution of diisopropylethylamine (DIPEA, 0.1 mL) and 3,6,9,12-tetraoxa-24,25-dithiatritriacontan-1-amine (18 mg, 0.036 mmol, 1.2 equiv.) in anhydrous DMF (5 mL) was then added to the reaction mixture. The reaction mixture was stirred overnight at room temperature. The solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel, eluting with step-wise gradients of CH₂Cl₂ to CH₂Cl₂-EtOAc to EtOAc-MeOH. Yield: 10 mg (18%). ¹H NMR (400 MHz, CDCl₃): δ 8.61 (dd, 2H), 8.53 (m, 4H), 8.01 (d, 2H), 7.95 (d, 1H), 7.61 (m, 4H), 7.48 (d, 1H), 7.33 (d, 2H), 3.69 (m, 12H), 3.38 (t, 2H), 2.78 (sept, 2H), 1.20 (m, 42H), 0.85 (m, 3H).

Synthesis of gold nanoparticles

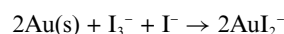
Synthesis of 4.5 nm TOAB-stabilized gold nanoparticles (TOAB-AuNP). Gold nanoparticles stabilised by tetraoctylammonium bromide (TOAB) in toluene ('naked particles') were obtained *via* a modification of the literature protocol.⁷³ A solution of HAuCl₄ (57 mg, 0.145 mmol) in 10 mL HPLC-grade water was added to a solution of TOAB (1.4 g, 2.56 mmol) in toluene (25 mL). The mixture was vigorously stirred until the tetrachloroaurate had been transferred into the organic phase. The aqueous phase became progressively colourless and the organic phase became orange. The organic phase was isolated, and 10 mL of a freshly prepared aqueous solution of NaBH₄ in water (40 mM, 6 equiv.) was added dropwise under vigorous stirring. The organic phase changed from orange to dark red indicating the formation of gold nanoparticles. Finally, the organic phase was isolated, washed with 0.1M HCl, saturated sodium carbonate solution, and brine, and dried over Na₂SO₄. The final suspension was characterised using UV/Vis spectroscopy, and compared to previously prepared samples⁴³ in order to confirm successful synthesis of 4.5 nm AuNPs. The concentration of nanoparticles and thiolate binding sites in the resulting suspension were estimated from the UV/Vis absorbance using literature data^{43,58} (for 4.5 nm TOAB particles: $\epsilon_{\text{particle}} = 6 \times 10^6 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{\text{binding-site}} = 1.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, number of thiol binding sites per particle ~ 380).

Synthesis of 13 nm citrate-stabilized gold nanoparticles (xCS-AuNP). Spherical citrate-stabilized gold nanoparticles (xCS-AuNP), with an average diameter of 13 nm were obtained following the Turkevich⁷⁴-Frens⁷⁵ citrate-reduction procedure. Briefly, a solution containing chloroauric acid (HAuCl₄, 0.3 mM, 180 mL) is brought to boiling, and an aqueous solution of sodium citrate (17 mM, 10 mL) is added rapidly. The mixture is stirred vigorously while under reflux, for an other hour. It is then allowed to cool at room temperature in the dark. The resulting suspension was characterised using UV/Vis spectroscopy. Comparison with previously synthesised particles^{17,18} confirmed successful synthesis of 13 nm xCS-AuNPs. The concentration of nanoparticles and thiolate binding sites in the resulting suspension were estimated

from the UV/Vis absorbance using literature data¹⁷ (for 13 nm xCS-AuNP particles: $\epsilon_{\text{particle}} = 2 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon_{\text{binding-site}} = 7.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, number of thiol binding sites per particle ~ 2800).

"Nano-waste" treatment

Because of concerns with the unknown fate and toxicology of nanoparticulate matter, waste containing gold nanoparticles was collected in a separate container. To this suspension was added a small amount of gold etchant solution (Transene "Gold etchant TFA"). Rapidly the red colour associated with the nanoparticulate gold disappeared, indicating complete conversion of AuNPs into gold salts. The gold etchant used is based on a solution of KI and I₂ which yields the net reaction



The final waste stream is thus completely devoid of nanoparticulate gold and its composition is identical to 'usual' microelectronic fabrication waste from gold etching procedures.

UV/Vis and fluorescence spectroscopy

UV/Vis absorption spectra and corrected fluorescence excitation and emission spectra were recorded on Cary 3E and a Spex Fluorolog 3 with a red-sensitive Hamamatsu R928 photomultiplier, respectively. Quantum yield measurements were performed on optically dilute solutions (absorbance well below 0.1) according to standard procedures.⁷⁶ Perylene Orange in toluene was used ($\Phi_F = 0.99$) as the reference,⁴⁸ since it has good spectral overlap with the compounds in this paper. Fluorescence decay times were measured using time correlated single photon counting (TCSPC) ($\lambda_{\text{exc}} = 323 \text{ nm}$ from a frequency-doubled cavity-dumped DCM dye layer) on a set-up that has been described elsewhere.⁷⁷

For characterisation of the gold nanoparticles in various solvents and their interaction with PMImSS, UV/vis absorption spectrometric measurements were performed using an optical fiber-based system (OceanOptics, USA) incorporating a USB4000-VIS-NIR CCD spectrometer and a LS-1 tungsten-halogen light source equipped with a BG34 colour correction filter. Fluorescence measurements were carried out on an other optical fiber-based system (OceanOptics, USA) using a QE65000 thermoelectrically cooled, back-thinned CCD spectrometer and a stabilised light-emitting diode (LS-450, 455 nm center wavelength) as an excitation source. Emission spectra from this spectrometer were not corrected for the wavelength dependence of spectrometer detection efficiency. The efficiency curve is expected to be rather flat in the region of PMImSS and PMImSS emission. Moreover, virtually no changes in the shapes of the emission spectra are found during the titration measurements, making the measurement of relative integrated intensities as needed for fluorescence titrations reliable.

Fluorescence correlation spectroscopy

The samples are illuminated by a pulsed excitation at 488 nm, obtained by frequency doubling a Ti-Sa femtosecond laser (repetition rate 80 MHz, pulse duration 100 fs, wavelength 796 nm) in a BBO crystal. The pulse duration of nearly 100 fs is controlled by a prism compressor and the beam is further cleaned by spatial filtering. The beam is the illumination source of an inverted confocal microscope. After reflection onto an appropriate beamsplitter

it covers the back aperture of a water immersion objective (N.A. = 1.2, 60×), which focuses the beam into the sample, about 15 μm above the coverslip in order to avoid the artefacts of the interaction of the studied species with the glass surface. The correction ring of the objective is carefully controlled to limit the spherical aberrations. The signal is collected in a backward configuration and filtered by a 30 μm diameter pinhole. The beam is then extended to match the core and the numerical aperture of the entrance of the collection fiber. As mentioned in the text, depending on the study, the spatial filter was in the path or removed to shorten the acquisition time and then the spatial filtering is ensured by the fiber core itself. The fiber is connected to a fast avalanche photodiodes (impulse response 50 ps). A spectral filter is added in the detection path to eliminate the laser diffusion. The avalanche photodiode signal is sent to a Single Photon Counting module (TimeHarp300, Picoquant). A home written computer program is used to numerically filter the bursts, to analyze the data in a given temporal window and to calculate the correlation of the data.

The samples were prepared by depositing a drop of the solution onto a clean cover slip. The data were acquired at air and at room temperature. The concentration is maintained low in order to work at the single particle level. Finally, the autocorrelation of the time-variant fluorescence intensity is obtained.

$$G(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I \rangle^2} \quad (5)$$

The brackets indicate time average. τ is the time lag. The Rayleigh background has been omitted in this expression. The autocorrelation function related to the Brownian diffusion of a single fluorescent species across the excitation volume, assuming a Gaussian–Gaussian observation volume reads as:

$$G(\tau) = 1 + g(\tau) = 1 + \frac{1}{N} \frac{1}{\left(1 + \frac{\tau}{\tau_D}\right) \sqrt{1 + \left(\frac{\omega_r}{\omega_z}\right)^2 \frac{\tau}{\tau_D}}} \quad (6)$$

N is the mean number of molecules diffusing simultaneously inside the focal volume, ω_r and ω_z the lateral and axial FWHM waists, respectively. τ_D is the diffusion time through the focal volume. The diffusion time equals $\tau_D = \frac{\omega_r^2}{4D}$, where D is the diffusion constant of the molecule, which depends on the viscosity of the sample, the hydrodynamic radius of the molecule and the temperature. If two non-interacting species are diffusing at the same time in the sample, the correlation function is the sum of two contributions of the same type as previously, with a weighting factor that is a function of the relative concentration of the two species and of their brightness. Blinking of the fluorescence of a species (e.g. as a result of protonation, complexation etc.) gives rise to an additional exponential decay factor⁵¹ In order to calibrate the set-up, we have used Rhodamine 6G as a reference in conditions similar to that used for the different samples. The diffusion coefficient of Rhodamine 6G has been determined accurately in water.⁵³

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