Phase Transfer and Polymer Coating Methods towards Improving Stability of Metallic Nanoparticles for Biological Applications

Mahmoud G. Soliman,[†,‡] *Beatriz Pelaz,*[*,†,‡] *Wolfgang J. Parak*[†,§] *and Pablo del Pino*[*,§]

[†] Fachbereich Physik, Philipps Universität Marburg, Marburg, Germany

[§] CIC biomaGUNE, San Sebastian, Spain

Abstract: This paper describes a general method to generate noble metal nanoparticles (NPs) with polymer coatings. One of the widely used approaches to stabilize NPs in aqueous solution involves wrapping NPs with amphiphilic polymers. This methodology has been extensively employed for polymer coating of small hydrophobic NPs (diameter of inorganic core < 20 nm), thereby enabling phase transfer of NPs from an organic solvent to aqueous solution. The polymer coating approach is herein extended to NPs originally synthesized in aqueous solution by a two-step method. Firstly, NPs are subjected to aqueous-to-organic phase transfer. The phase transfer protocol is demonstrated for NPs made of different materials (Au and Ag), sizes (up to 100 nm) and shapes (spheres, rods, and flat-triangular prisms). Secondly, NPs are coated with an amphiphilic polymer. The colloidal stability of a variety of the newly designed NPs is assayed

against different media of biological relevance. In preliminary cellular studies, the biocompatibility of polymer coated Au NPs is investigated in different cell lines.

INTRODUCTION

Current bottom-up chemical methods allow to synthesize NPs with a large range of possible size, shape and composition. This is possible with a high degree of control in terms in monodispersity and size distribution.[1-4] Synthesis can be either carried out in organic solvents or in aqueous solution. In addition to being able to control the physicochemical properties of the end products by chemical methods, colloidal stability of NPs in physiological media is critical if these are to be used in biological applications.[5] This implies that NPs should be stable in aqueous media with high ionic strength, high protein concentration, and a specific range of pH values. In contrast to NPs synthesized in aqueous media, methods to stabilize hydrophobic NPs require a phase transfer step which can be effectively achieved by different methods, such as the replacement of the hydrophobic chains by hydrophilic ones (ligand exchange),[6] or the use of amphiphilic polymers.[7] Two main advantages of the latter compared to other existing methods are to be highlighted. Firstly, the original surface of the inorganic material (be it metal, semiconductor or metal oxide) is *a priori* not affected and therefore, the physical properties of the core such as quantum yield, absorption, scattering or magnetism are in general less disturbed as compared to ligand-exchange procedures. Secondly, the NPs surface is uniformly wrapped with a common polymer, which translates into the generation of a common surface chemistry for different materials. Notably, the later allows to produce NP models with similar surface chemistry, yet with different physicochemical properties due to different inorganic cores. Magnetic, semiconductor and metallic NPs, with different size, shape and composition, have been successfully stabilized by the later. [7,8 9-11]

In the literature, the number of works which attempt to evaluate the impact of NPs in cells, or even complex organisms, is overwhelming.[12] Yet correlating physicochemical properties of NPs and their biological fate and impact is still not straightforward.[5] This is partly so because of the enormous variety of NP models employed to date, which will even continue to grow as new methods and materials arise. Besides the variety of NP models, it appears clear that the biological fate of NPs is highly influenced by the NPs' surface chemistry, which has been also demonstrated to be very important in the formation of the so-called protein corona.[13] As proven already with different NP models, varying the coating of NPs greatly influences several important biological parameters such as cellular uptake, NP localization inside cells, toxicity, circulation, biodistribution, protein corona, *etc*. The design of the surface of NPs represents a key step towards multifunctional NPs, which are typically achieved by anchoring distinct molecules of biological relevance onto the NPs' surface. The biological fate of NPs is also determined by the protein corona. Ultimately, the protein corona formation can be responsible for different results observed using similar nanosystems.[14] The use of amphiphilic polymers to coat NPs would eliminate the surface factor from the equation, providing thereby a NP model in which is possible to determine the role of one parameter at a time, such as size, vectors, stiffness, ions release, *etc.* Additionally, this methodology provides NPs with a high colloidal stability in cell media, and against high salt concentration, and in a broad pH range.[8,15] These reasons make this technique highly interesting to produce NP models for biological applications. However, to date, this methodology has been traditionally limited to small NPs, typically with inorganic cores with diameter < 20 nm, originally synthesized in organic solvent and therefore, many bio-relevant NPs originally synthesized in aqueous media (*e.g.* anisotropic plasmonic NPs) have not benefited from this technique. In order to apply this methodology to such NPs (*e.g.* CTAB-coated gold

nanorods, where CTAB stands for the cationic surfactant cetyltrimethylammonium bromide), a previous phase transfer to an organic solvent should be performed.

For the aqueous-to-organic media transfer of Au NPs the ligand exchange technique has been applied by using aliphatic chains with a terminal thiol group such as dodecanethiol (DDT),[16] or by using chains with a terminal amine group such as dodecylamine (DDA) or hexadecylamine (HDA).[17] The main limitation of the ligand-exchange method is again the size, as it typically works with NPs of diameters smaller than *ca.* 20 nm, in the case of free-surfactant capped NPs. To our knowledge, only few examples with NPs > 20 nm have been described in the literature.[18,19] The need of surfactants to transfer large particles is obvious according to the published works in this direction, in which CTAB or similar surfactants are typically involved, be it as additives for the phase transfer process or in the NPs synthesis.[16,20] For anisotropic Au NPs, the phase transfer of gold nanorods using DDT has been used, aiming washing out the excess of the cytotoxic surfactant CTAB. The toxic effects of CTAB have been extensively proved.[21,22] In order to decrease the amount of CTAB, a round trip from aqueous phase to organic media and again to aqueous media has been previously reported.[23-25]

Herein, we report on a straightforward method which allows for aqueous-to-organic phase transfer of relevant Au NPs with different sizes (inorganic cores with diameters of 25, 50, 60 nm up to nanorods with 90 nm in length) and shapes (spherical and rods). As a proof-of-concept to extend this methodology to an additional shape and another plasmonic material, Ag nanoprisms were also successfully polymer coated using equivalent methods as for Au NPs. The basis of the proposed method relies on a pre-stabilization step using short (low molecular weight) polyethylene glycol (PEG) chains. A recent work in which long alkyl-PEG chains has been described as ligands to transfer nanorods to the organic phase using centrifugal forces has been

recently reported.[26] NPs smaller than 20 nm were transferred using mixed chains containing an aliphatic domain and a hydrophilic PEG chain, which allow to get NPs colloidally stable in both solvents.[27] In the present work, short PEG chains were used as pre-stabilizers and active agents for the phase transfer of NPs. The pre-stabilization step was required to warrant colloidal stability in the phase transfer process. In case the NP size > 15 nm, *i.e.* inorganic cores with diameters larger than 15 nm, ligands such as DDT or HDA yielded unsatisfactory results. Thus, as an alternative to toxic surfactants such as CTAB or didecyldimethylammonium bromide (DDAB), the use of short PEG chains was investigated. PEGylated NPs can be driven to an organic phase (chloroform) containing DDA under vigorous stirring at room temperature. The time required depended on the original coating of the NPs. For instance, CTAB-coated gold nanorods (GNRs) required longer times than citrate capped Au NPs. The use of ethanol helped to complete the transfer faster.[28] DDA can then intercalate with the amphiphilic polymer dodecyl-grafted-poly-(isobutylene-alt-maleic-anhydride) (PMA), enabling aqueous transfer for different colloids. In order to enhance the stability of the polymer-coated NPs in cell media, further PEGylation was employed. Finally, stability and toxicity studies are shown as examples to illustrate the potential of this method to provide NP models for biological applications.

EXPERIMENTAL SECTION

Materials. Prior to use, all glassware was washed with *aqua regia*, and rinsed thoroughly with Milli-Q water. All the chemicals were used as received. For the synthesis and modification of the NPs, hydrogen tetrachloroaurate (III) hydrate was purchased from Strem Chemicals; sodium citrate, silver nitrate, CTAB, sodium borohydrade, ascorbic acid, hydrogen peroxide, dodecylamine, poly-(isobutylene-maleic-alt-anhydride), hydrochloridric acid, sodium borate, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and sodium oleate were purchased from Sigma-

Aldrich. All the different polyethylene glycol polymers (PEG) were obtained from Rapp-Polymere. For the stability studies, phosphine buffer saline (PBS) was purchased in Biochrom and DMEM, penicillin, streptomycin and L-glutamine from Sigma Aldrich. They were used as received. For the viability tests, resazurin was purchased from Sigma Aldrich, and was used as received.

Synthesis of NPs. Citrate-capped spherical gold NPs with diameter of *ca.* 25 nm, 50 nm and 60 nm, in the following referred to as 25-GNPs, 50-GNPs and 60-GNPs, respectively, were synthesized using a seed-growth method reported elsewhere, *cf.* supporting information (SI, section 1).[1] CTAB-capped GNRs with plasmon band (*i.e.* localized surface plasmon resonance – LSPR) centered at *ca.* 850 nm and 1100 nm, in the following referred to as 850-GNRs and 1100-GNRs, respectively, were prepared by using the seed-mediated growth method recently published by Murray and co-workers, which allows to produce GNRs with a large variety of aspect ratios. We choose as NP models[2,29]: short 850-GNRs (low aspect ratio) and long 1100-GNRs (high aspect ratio), *cf.* SI (section 1). Citrate-capped silver nanoprisms (*ca.* 60 nm in length) with plasmon band centered at *ca.* 600 nm (in the following referred to as AgNPRs) were obtained by the reduction of silver nitrate with H_2O_2 and sodium borohydride in aqueous solution, as reported elsewhere,[4] *cf.* SI (section 1).

NP stabilization: ligand exchange with PEG. After synthesis, the GNPs were cleaned from the free citrate by centrifugation using centrifugal filters (20 mL, 100 kDa, Millipore) at 110 g for 5 min (one time). The GNRs were cleaned by centrifugal precipitation at 7080 g for 25 min (one time). In all cases the NPs were diluted in MilliQ water (1.37, 0.41, 0.13, 5.1 and 0.74 nM for 25-, 50-, and 60-GNPs, 850- and 1100-GNRs, respectively) and stabilized by mPEG-SH (CH_3O-PEG-SH, M_w= 750 Da, (Rapp Polymere)) dissolved in MilliQ water. 30 µL of NaOH (100 mM)

per mL of GNPs, and 10 μL of NaOH (1 M) per mL of GNRs were added to increase the pH to *ca.* 10, aiming to increase the reactivity of the thiol group.[3] The stoichiometric ratio of PEG molecules to NP (C_{PEG}/C_{NP}) was $5 \cdot 10^5$. The solution was mixed with stirring (400 rpm) overnight. Please note that $5 \cdot 10^5$ is the maximum amount required to transfer successfully the "bigger" NPs. To transfer small particles using lower PEG:NP molar ratios are also possible, *cf.* Table S2 and section 2 in the SI for further details.

Phase transfer. Upon PEG stabilization of the NPs, *i.e.* PEGylation, they were transferred from aqueous media to organic solvent (chloroform) by using DDA (0.75 M) dissolved in chloroform with strong magnetic stirring (1200 rpm). The transference for "small" NPs was also feasible by using lower concentrations of DDA, *cf.* Table S2 in the supporting information. The phase transfer requires 12 h for GNPs, and 4 days for GNRs. Stirring has to be strong enough to mix the two phases perfectly. After this, the water was removed and the samples were washed twice with chloroform by precipitation. To clean 25-GNPs and 1100-GNRs, 8960 g during 30 min was used. Meanwhile to clean 50 and 60-GNPs and 850-GNRs, 2240 g for 40 min were selected. Then, the supernatant was removed. GNPs and GNRs were re-dispersed in chloroform again prior to polymer coating.

Polymer Coating. The NPs were water transferred using the polymer coating technique as previously described in previous reports.[7,30] Briefly, polymer coating for GNPs and GNRs was done by dissolving an appropriate amount of polymer monomers per NP surface unit ($R_{p/area}$ [nm^2]). In all the samples we added 3000 monomers of poly(isobutylene-*alt*- maleic anhydride) modified with dodecylamine (hereinafter referred to as PMA) dissolved in chloroform per nm^2 of NPs. For details about the synthesis of the amphiphilic polymer used here we refer to the previous work of Lin *et al.*[7]. Briefly, the PMA was synthesized by grafting dodecylamine onto

the poly(isobutylene-alt- maleic anhydride) backbone through spontaneous amide linkage, which converts one maleic anhydride into one corresponding amide and one free carboxylic acid. In the amphiphilic polymer used in our work, 75% of its maleic anhydride rings have been reacted with dodecylamine, leaving 25% of its anhydride rings intact. To increase the stability the use of a crosslinker has been also previously proposed.[7,31] We tested two different crosslinkers, bis(6-aminohexyl)amine and polyethylenimine (branched, M_w = 800 Da). More details about the use of the crosslinker can be found in the SI. After addition of the polymer, the solvent was slowly evaporated using a low-pressure system, until the sample was completely dry. Then, more chloroform was added and the drying process was repeated. The NPs were quickly dissolved in 0.1 M NaOH, which hydrolyzes the remaining maleic anhydride rings, leaving 2 carboxylic groups per newly opened anhydride ring. Then, the solution was filtered through a 0.22 μm syringe filter. After this, the NPs were precipitated by centrifugation (same conditions as for the phase transfer) twice, in order to remove the residual empty polymer micelles[32]. Washing out empty micelles and unwanted byproducts is also possible by gel electrophoresis. After purification, the GNPs and GNRs were re-dispersed and kept in MilliQ water. Extinction coefficient values and further calculations for the polymer coating method can be found in the SI.

Surface modification of PMA-coated NPs: PEGylation. The surface of PMA-coated gold NPs was modified with mPEG-NH_2 (CH_3O-PEG-NH_2, Rapp Polymere). The carboxylic groups of the polymer (note: generated by grafting of dodecylamine and hydrolysis of the anhydride rings) and the amine groups of PEG were cross-linked by using EDC chemistry in MilliQ water according to the following table:

Table 1. Conditions for PEGylation of PMA-coated NPs.

NPs	C_{EDC}/C_{NP}	mPEG-NH_2	mPEG-NH_2

		(M_w, kDa)	(C_{PEG}/C_{NP})
25-GNPs	$5 \cdot 10^6$	0.75	$6 \cdot 10^6$
45-GNPs	$30 \cdot 10^6$	5	$3 \cdot 10^6$
60-GNPs	$30 \cdot 10^6$	10	$3 \cdot 10^6$
850-GNRs	$9 \cdot 10^6$	5	$7.5 \cdot 10^5$
1100-GNRs	$9 \cdot 10^6$	10	$7.5 \cdot 10^5$

After PEGylation, NPs were cleaned from free PEG by applying an electric field of 10 V/cm for 1 h in an electrophoresis tank. The agarose bands with the NPs were transferred to a dialysis membrane (molecular cut-off (MWCO) = 50 kDa) and then, NPs were extracted from the agarose by electrophoresis using the conditions above described. Lastly, NPs were centrifuged once using the conditions above described; the supernatant was removed and the NPs were re-dispersed and kept in MilliQ water.

Characterization. All NPs were characterized by UV/Vis spectroscopy (Agilent 8453 spectrometer), Dynamic Light Scattering (DLS) and Laser Doppler Anemometry (LDA) (Nanosizer, Malvern), electron microscopy (Jeol 1400 plus), and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Agilent 7700 series ICP-MS).

Stability studies. In order to evaluate the NP stability against media of biological relevance, solutions with the same concentration of NPs were dispersed in the following 8 different media: 1) water (Milli Q), 2) phosphate buffer saline (PBS, Biochrom), 3) Dubelccos´s modified Eagle´s media (DMEM, Sigma Aldrich), 4) PBS 1% penicillin and streptomycin (P/S, Sigma Aldrich) and 1 % Glutamine (L-Glu, Sigma Aldrich), 5) DMEM 1% P/S and 1% L-Glu, 6) 800 μM bovine serum albumine (BSA, Sigma Aldrich) in PBS, 7) 800 μM BSA DMEM 1% P/S, 1% L-Glu and 8) DMEM 1% P/S, 1% L-Glu and 10% fetal bovine serum (FBS, Biochrom).[15] Their hydrodynamic radii and UV-Vis spectra were monitored at different time points, from 0 h to 3 days.

Cytotoxicity evaluation: Resazurin test. $5 \cdot 10^3$ tumoral human cells (HeLa) and $10 \cdot 10^3$ mouse fibroblasts (3T3) were incubated for 24 h in a 96-well plate at 37 ºC and 5% CO_2 with complete DMEM media (10% FBS, 1%P/S and 1% L-Glu). After this period, different concentration of NPs dispersed in cell media were added. Three measurements were done for each concentration. Then, after another 24 h of incubation at 37 ºC and 5% CO_2, cells were washed 3 times with PBS. 100 µL from a 10% solution of resazurin (7-Hydroxy-3*H*-phenoxazin-3-one 10-oxide; Sigma Aldrich) in cell media was added into the wells. After 3 h of incubation, the fluorescence spectra (range from 572 to 650 nm) were recorded in a fluorescence spectrometer (Horiba Jobin), upon excitation at 560 nm. This test is based on the irreversible oxidation of resazurin to the pink and highly fluorescent resorufin. To analyze the data, the average of the background was subtracted from the maximum value. To get the percentage of viable cells, control wells were just cells were incubated equivalently, were considered as 100% viable cells.[8]

RESULTS AND DISCUSSION

The main aim of this work is to establish methods for the phase transfer and polymer coating of noble metal NPs with diameters larger than 20 nm and high anisotropy. The polymer coating procedure can be thus extended to a wide variety of NPs with numerous bioapplications. The realization of this approach requires a water-chloroform-water round trip for the NPs, *cf.* Figure 1.

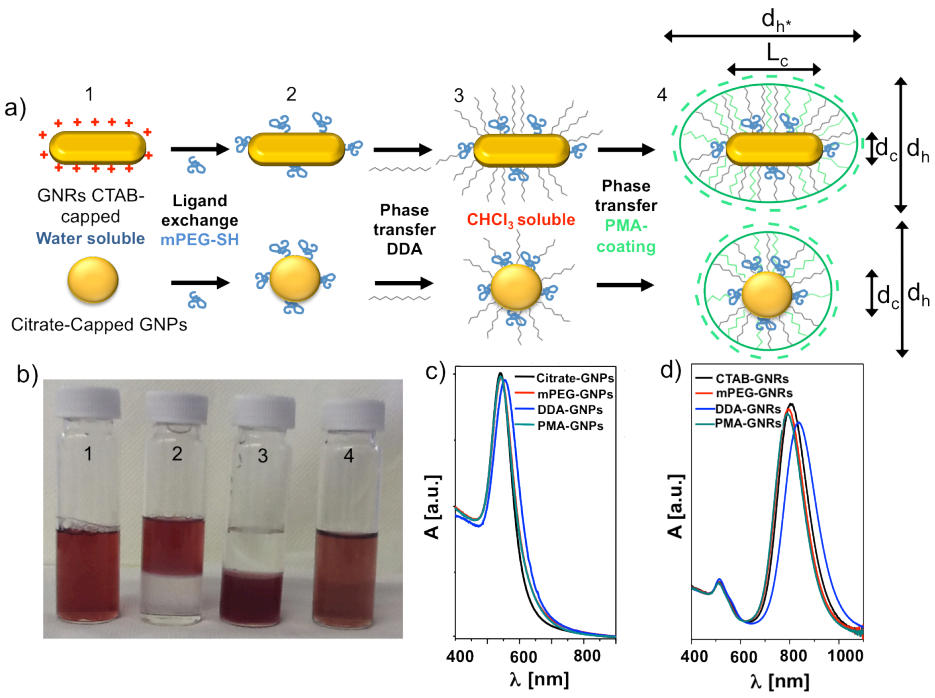

Figure 1. a) Schematic representation of the coating strategy for both spherical GNPs and GNRs. b) GNRs in water after their synthesis (1), PEGylated GNRs before the phase transfer (2), in chloroform after the phase transfer using DDA (3), and after their polymer coating with PMA (4). Notice that we cannot rule out the presence of short PEGs intercalated within the DDA coating. UV/Vis spectra corresponding to all the steps of the process for 60-GNPs (c), and 850-GNRs (d). See SI for the UV/Vis spectra of the other NPs, section 2).

Firstly, a pre-stabilization of the NPs in the aqueous phase was required. Indeed, without this pre-stabilization step, we were unable to transfer bare NPs with sizes > 15 nm. Small NPs and surfactant-capped NPs (*e.g.* CTAB capped or TOAB capped) did not require this pre-stabilization step, but the use of the PEG chains improves the yield of the transference in all of the cases. Short chains of PEG (750 Da) were employed as pre-stabilizing agent. The selected heterofunctional chains bear a thiol group in one end and a methoxy group in the other. Notice that we used thiolated molecules because in this study, Ag and Au NPs were our targets (Figure

2), which can readily bind thiolated molecules. This chain, in particular, was selected due to its length and the non-charged methoxy end.

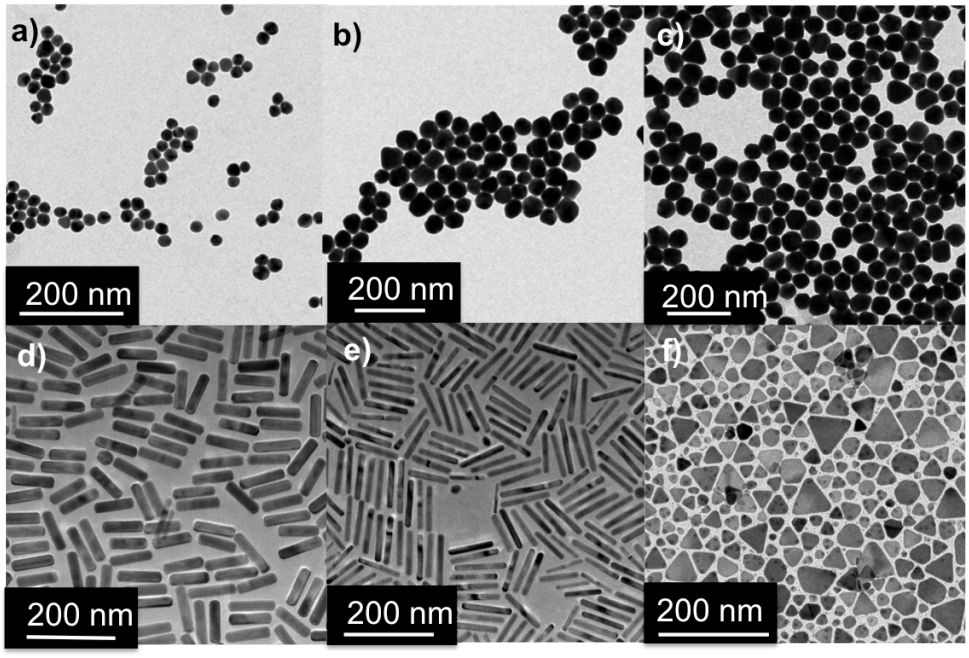

Figure 2. Transmission electron microscopy (TEM) micrographs of the NPs: spherical GNPs a) 25-GNPs, b) 50-GNPs, and c) 60-GNPs, and anisotropic NPs: d) 850-GNRs, e) 1100-GNRs, and f) AgNPRs

Notice that the approach here described focused on metallic NPs synthesized in aqueous solution and thus, alternative end-terminal groups might be more efficient to pre-stabilize with PEG other NPs in aqueous solution. For other materials than Au or Ag, such as metal oxides (*e.g.* Fe_3O_4, ZnO, TiO_2, NiO, MnO, CeO, *etc*.) or QDs, end-terminal groups other than thiol might be required. For instance, one might speculatively hypothesize that PEG-silane, PEG-siloxane, PEG-phosphine oxide or PEG-phosphoric-acid might be used for pre-stabilization of a variety of metal oxides.[33-36] By a more general approach, poly(histidine)-PEG could be used to pre-stabilize carboxyl-terminated NPs (independently of the inorganic core), as recently shown by Wegner *et al*. for poly(histidine)-derivatized biomolecules and QDs.[37] Yet, in case of "water-soluble" CdTe

QDs[38] thiolated PEGs might efficiently pre-stabilize them as in the case of Au and Ag. Although as previously stated the use of the pre-stabilization step is required for "big" metallic NPs, adapting the proposed method to other materials and PEGs (if needed) will need further optimization due to different binding affinities.

As previously explained, the main aim is to coat the NPs with PMA as a model polymer, although other polymers and PMA modified with functional molecules (dyes, chelators, SERS tags, *etc.*)[39] have been successfully employed in previous works. This actually illustrates the versatility of the method proposed in terms of achieving multifunctional NPs. We anticipated that the PEG chains would stabilize the NPs and due to their short length, they would not prevent the interaction of the aliphatic-PMA domain with the aliphatic chains that will be introduced on the NPs later on in the organic phase. The amphiphilic nature of PEG chains is well known,[40] and it is supported by the dual solubility either in aqueous solution or organic solvents, such as chloroform. Taking advantage of this double behavior, PEG chains were expected to work as stabilizers and phase transfer helpers. The modification of NPs with PEG is a straightforward procedure extensively used to stabilize NPs.[3,41,42] Once stabilized with PEG, the NPs were ready to be transferred to the organic phase.

In the early stage of the development of the proposed method, different combination of solvents and aliphatic chains were tested (see Table S2 in the SI). The combination of chloroform and DDA was found to be the best combination for our purposes. To successfully transfer the NPs, it was only required to let the two phases interact (aqueous and organic) by using vigorous stirring. DDA-capped NPs presented high stability in organic media and their absorption bands remained very similar to the original ones. Please notice that we cannot rule out the presence of short PEG intercalated within the DDA coating. As expected, only a small red shift was observed due to the

change of the NPs environment (see Figure 3, and section 2 from SI). The DDA-capped NPs were precipitated by centrifugation to remove free, unbound ligands. Although our main aim is towards biological purposes, having plasmonics NPs in organic solvent is very interesting for other applications (*e.g.* the production of thin films, which contain NPs, or nanocomposites). The transfer procedure worked similarly for all the NPs reported in this work, the only difference resided in the required time for the transfer. This time depended on the nature of the original ligand on the NPs surface. The transfer time could be shortened by the addition of ethanol to improve the contact between the interfaces.[28] It is interesting to highlight that some NPs could be transferred to the organic phase just by the use of the PEG chains. Nevertheless, the yield of the transferred NPs was much lower compared with the yield obtained using DDA (almost 100%; this yield was determined by ICP-MS, see Table S6 in the supporting information). Recently, Alkilany and co-workers have proposed a protocol to aqueous-to-organic phase transfer of gold NPs which combines thiolated PEG chains (with molecular weight > 1 kDa) and methanol.[19]

Once the NPs were dissolved in chloroform, the classic polymer coating technique was applied. To this end an optimization step was done regarding the amount of polymer needed to coat the NPs (see supporting information, section 3). In agreement with previous work,[7] the amount of polymer required is expressed in the number of monomers *per* effective NP surface ($R_{P/area}$). The NPs used here required higher $R_{P/área}$ values as compared to typical values previously reported for smaller NPs. Also in comparison with smaller NPs, the polymer coating procedure worked best when the solvent was removed using reduced pressure conditions, *i.e.* the pressure was kept high and the temperature in the bath was also high (60-70 ºC). The high colloidal stability of PMA-coated NPs typically allows for cleaning steps, both by using gel electrophoresis (see supporting information, Section 4) and by centrifugation. Aiming to achieve a higher stability (stable in

297 water or biological media for months), the use of crosslinkers was also tested. Crosslinkers were
298 meant to act as stapler to "polymerize" the different polymer molecules which wrap the NPs.
299 Initially, the short molecule bis(6-aminohexyl)amine was used as crosslinker as previously
300 reported.[7,31] In this case, probably due to the bigger size of these NPs, this short molecule did not
301 improve the stability of the NPs and therefore, we tested another crosslinker: poly(ethylenimime)
302 (PEI, 800 Da). After testing the stability of the NPs coated with PMA only, the short crosslinker
303 and PEI, we determined that a 0.75% of PEI improved the stability of the NPs (see the data in the
304 supporting information, for nanorods) in complete cell media (and for all the NPs, section 6 from
305 SI).
306 In order to further improve the colloidal stability of the NPs, stabilization with PEG (mPEG-NH_2
307 chains), *i.e.* PEGylation, was carried out by 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
308 (EDC) chemistry.[33] The molecular weight of the PEG chains was varied between the different
309 samples (from 0.75 to 10 kDa). In general, the molecular weight of the PEG was increased as the
310 size of the inorganic core increased. The stability of these NPs was tested against 8 different
311 media, which are important in biological applications.[15] The stability was tested for time points
312 up to 3 days by dynamic light scattering (DLS) and UV/Vis spectroscopy (SI section 7). The
313 stability role of the crosslinker (PEI) and PEGylation were also tested using UV/Vis
314 spectroscopy (see Figure 3, and section 7 from SI). To compare the effect on the stability of the
315 individual elements used to wrap the NPs, the same concentration of NPs coated with PMA only
316 (Figure 3a), coated with PMA and the crosslinker (PEI, Figure 3b), and PEGylated-PMA-PEI
317 coated NPs (Figure 3c) were incubated in complete cell media (media 8), and the changes of
318 their UV/Vis spectra were monitored over time, from time 0 to 3 days. The addition of PEI
319 prevents slightly the initial broadening of the plasmon band, which disappears with the time,

presumably, due to absorption of proteins onto the NPs surface. This broadening is completely prevented by the PEGylation of the NPs. In all the cases the colloidal stability of the NPs is very high, even after 3 days (see SI for the rest of the stability tests).

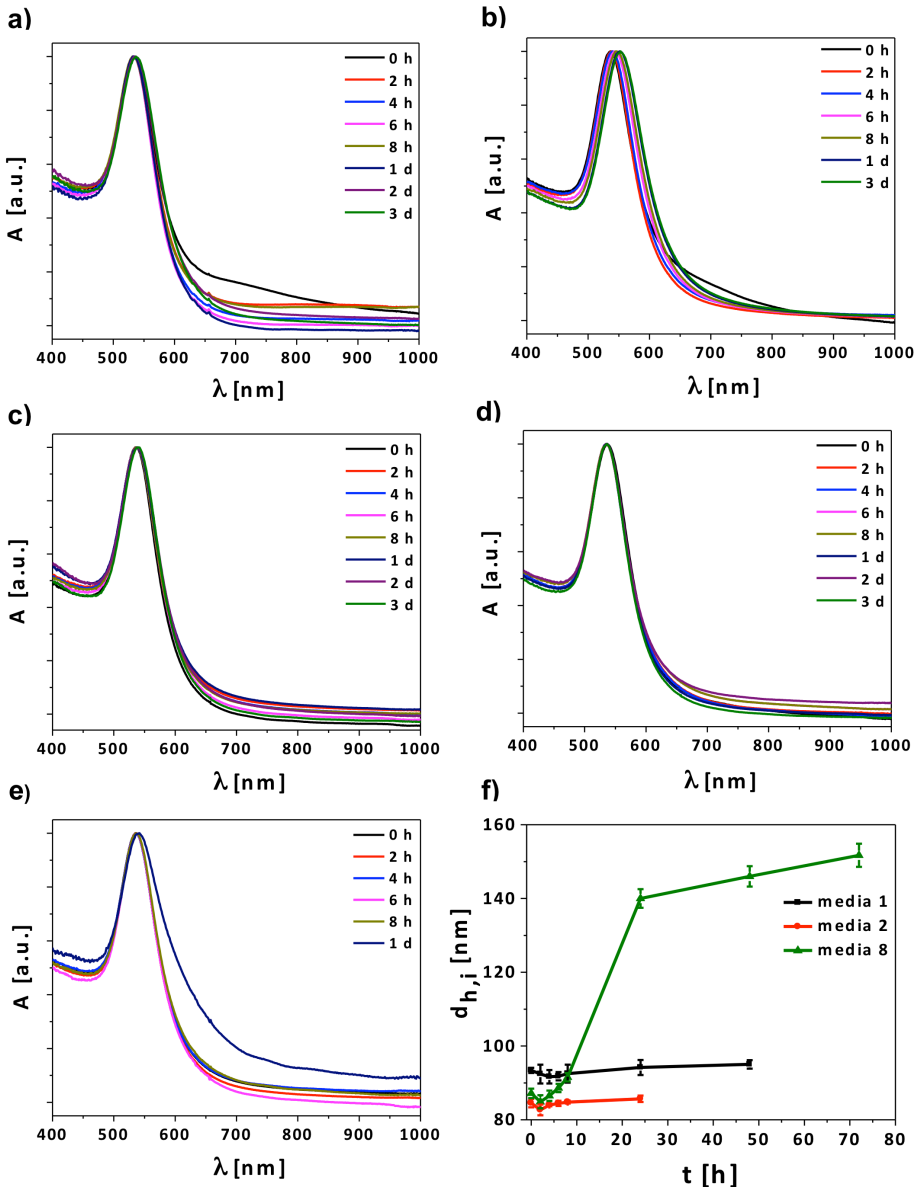

Figure 3. Stability tests against complete cell media of 50-GNPs followed by UV-Vis spectroscopy *versus* time of NPs stabilized with: a) just with PMA, b) PMA-PEI, c) PMA-PEI PEGylated. Stability of PEGylated PMA-PEI 50-GNPs in water (d) and in PBS (e). Stability

followed by DLS for PEGylated PMA-PEI 50-GNPs in water (medium 1), PBS (medium 2) and complete cell media (medium 8).

The stability of PEGylated NPs against the 8 above described media was tested. Our results indicated that the stability of all the NPs in the media containing proteins (6, 7 and 8) was greatly improved, as compared to their stability in free-protein media (2, 3, 4 and 5) (Figure 3c, e and f). In some of the free-protein media, NPs were not stable for longer than 24 h. This indicates that the presence of salt in the media compromises the NP stability due to the screening of the NPs' charge. Our data seem to indicate that the salt effect is counteracted by the absorption of proteins, *i.e.* the protein corona.[43] The protein absorption was evidenced by a redshift in the LSPR (Figure 3b) of the NPs in the UV/Vis spectra, and by an increment in hydrodynamic radius, as resulted from the DLS analysis. (See section 7 from SI for all the results).

Notably, the increment of the hydrodynamic radius observed for media 6 and 7, which contain BSA (0.8 mg/mL), differs from the increment observed for medium 8, which contains FBS (10%). Our results allow us to speculate whether these changes might be due to the different concentrations of BSA in the corresponding medium, or due to the presence of different proteins. Indeed, the concentration of BSA in medium 8 was *ca.* 2.3 mg/mL, considerably higher than in media 6 and 7 (*i.e.* 0.8 mg/mL). Protein binding, and thus the size and composition of the protein corona, is highly influenced by the concentration of protein species.[43] The major constituent of FBS is BSA. Indeed, it has been previously proposed that the main component of the protein corona is BSA.[44] However, our data do not allow to rule out that the differences between media 6/7 and 8 are instead due to the absorption of other proteins than BSA and/or due to a PEG conformational change. For media 6 and 7 the trend is very similar, except for 25-GNPs, which has a behavior closer to that in medium 8. Our qualitative studies do not allow to clarify this out-

of-trend behavior of 25-GNPs, which might be due to its different size (also, curvature radius), different PEG length (750 Da) and/or different protein corona. In general protein absorption is a time dependent process.[44,45] For all the other NPs in media 6 and 7, the size increment is observed immediately after the addition of the medium, that is, there is no increase over time. In contrast, in case of medium 8, the hydrodynamic radius increases significantly after *ca.* 10 h until reaching a plateau at *ca.* 20 h, which indicates that longer incubation times are required. These results are supported by the previous work of Maiorano and coworkers.[46] They used citrate-capped Au NPs and evaluated the protein corona evolution along the time. They found that using DMEM supplemented with FBS, the hydrodynamic radius of the NPs needs more than 50 h to reach a plateau. Note that this time is comparable with our results. These qualitative results demonstrate that even though the NPs were saturated with PEG, as indicated by their ζ–potential values and their electrophoretic motilities (see SI, section 4 and Table S7), the unspecific absorption of proteins was not avoided. These results will lead to further studies regarding the PEG conformation on this kind of surfaces.

Aiming at proving that this double round trip of the GNRs can be used to remove CTAB,[47] or at least to minimize CTAB-release from the NP surface, the toxicity of the GNRs was evaluated. In this case, 3T3 fibroblasts cells and HeLa cells were incubated with increasing amounts of PMA-coated GNRs (plasmon band placed at *ca.* 850 and 1100 nm) for 24 h. For completeness, the impact on cell viability of the rest of the Au NPs was also investigated. In all the cases no acute toxicity features were observed (Figure 4). For the GNRs no acute toxicity was observed in both cell lines when working with concentrations up to 2.5 nM (see Figure 4b). To test viability, the resazurin test was chosen. By this test, the mitochondrial respiration was evaluated, which can be in general related with the cell viability. Interestingly, we found that the viability trend is size-

dependent, as 60-GNPs started to exhibit toxicity at 0.2 nM (263 µg/mL), *cf.* supporting information section 8. However, this trend should be further investigated and confirmed by a multiparametric methodology.[48]

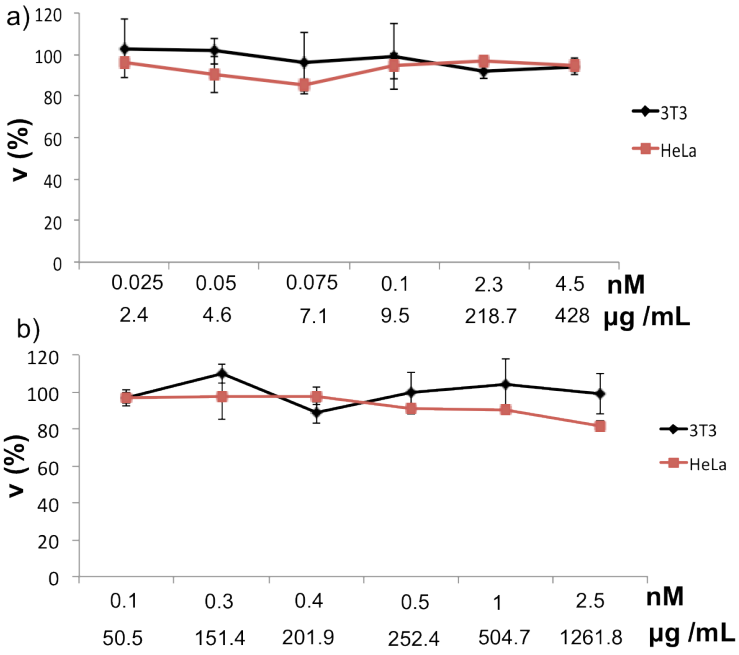

Figure 4. Viability V results for a) 25-GNPs and b) 850-GNRs after 24 h of incubation for 3T3 and HeLa cells.

CONCLUSIONS

This work reports on a phase transfer strategy for NPs with different sizes, shapes and materials. The use of short PEG chains (750 Da) as pre-stabilizers of NPs for their transfer to organic solution is reported. This approach is very versatile, as it allows the transference of NPs within a long range of sizes to the organic phase. Nowadays, different functionalized PEG molecules are commercially available, which makes this approach accessible for any kind of laboratory. Additionally, the use of PEG as stabilizer of NPs has been widely reported and thus, to adapt this strategy to other materials should be very straightforward. The transferred NPs keep their

plasmonic properties and a good colloidal stability after their transfer. Notably, the transfer works with big volume samples (more than 500 mL), and the NPs can be dried and redispersed again, keeping their optical properties. The transferred particles were successfully coated with the amphiphilic polymer PMA, which allows to form equivalent coatings for different NPs. This is very interesting for comparing the interaction of different NPs with living cells. This methodology has been typically restricted to aliphatic-coated NPs, typically with inorganic diameters < 20 nm, in organic media. Here we have demonstrated that a variety of "big" NPs, after polymer coating, exhibit high colloidal stability in biological media containing high protein concentration. The coated NPs were found to be under realistic concentrations without acute toxicity for either mouse fibroblasts (3T3 fibroblast) or human cells (HeLa).

ACKNOWLEDGEMENTS.

The authors are grateful to Marta Gallego for technical assistance with electron microscopy.

ASSOCIATED CONTENT

Supporting Information. Additional data for synthesis of NPs, phase transfer optimization, polymer coating, PEGylation, NP characterization, enhancing the stability, stability assays, and toxicity tests. This material is available free of charge via the Internet at http://pubs.acs.org."

AUTHOR INFORMATION

Corresponding Author

* Beatriz Pelaz (beatriz.pelazgarcia@physik.uni-marburg.de) and Pablo del Pino (pdelpino@cicbiomagune.es)

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

Funding Sources

Parts of this work were supported by the German Research Foundation (DFG, GRK 1782 to WJP), and the MIMECO (MAT2013-48169-R, NanoFATE to WJP and PdP). B.P. thanks the Alexander von Humboldt Foundation for a fellowship and MGS thanks to the Youssef Jameel Foundation for an PhD fellowship.

ABBREVIATIONS

NPs, nanoparticles; CTAB, cetyltrimethylammonium bromide; PEG, polyethylenglycol; DDT, dodecanthiol; HDA, hexadecylamine; DDAB, Didecylammonium bromide; DDA, dodecylamine; PMA, dodecyl-graft-poly-(isobutylene-maleic.alt.anhydride); GNPs, gold nanoparticles; GNRs, gold nanorods; LSPR, localized surface plasmon band; NPRs, nanoprisms; TOAB, tetraoctylammonium bromide; PEI, polyethylenimine; PBS, phosphate buffer saline; DMEM, Dubelcco´s modified Eagle´s media; P/S, penicillin/streptomycin; L-Glu, L-glutamine; DLS, dynamic light scattering.

REFERENCES

1. Bastus, N. G.; Comenge, J.; Puntes, V., Kinetically Controlled Seeded Growth Synthesis of Citrate-Stabilized Gold Nanoparticles of up to 200 nm: Size Focusing versus Ostwald Ripening. *Langmuir* **2011,** 27, (17), 11098-11105.
2. Ye, X.; Jin, L.; Caglayan, H.; Chen, J.; Xing, G.; Zheng, C.; Doan-Nguyen, V.; Kang, Y.; Engheta, N.; Kagan, C. R.; Murray, C. B., Improved Size-Tunable Synthesis of Monodisperse Gold Nanorods through the Use of Aromatic Additives. *ACS Nano* **2012,** 6, (3), 2804-2817.
3. Pelaz, B.; Grazu, V.; Ibarra, A.; Magen, C.; del Pino, P.; de la Fuente, J. M., Tailoring the Synthesis and Heating Ability of Gold Nanoprisms for Bioapplications. *Langmuir* **2012,** 28, (24), 8965-8970.

4. Zhang, Q.; Li, N.; Goebl, J.; Lu, Z.; Yin, Y., A Systematic Study of the Synthesis of Silver Nanoplates: Is Citrate a "Magic" Reagent? *Journal of the American Chemical Society* **2011,** 133, (46), 18931-18939.
5. Rivera Gil, P.; Jimenez de Aberasturi, D.; Wulf, V.; Pelaz, B.; del Pino, P.; Zhao, Y.; de la Fuente, J.; Ruiz de Larramendi, I.; Rojo, T.; Liang, X.-J.; Parak, W. J., The Challenge to Relate the Physicochemical Properties of Colloidal Nanoparticles to Their Cytotoxicity. *Accounts of Chemical Research* **2013,** 46, (3), 743-749.
6. Salas, G.; Casado, C.; Teran, F. J.; Miranda, R.; Serna, C. J.; Morales, M. P., Controlled synthesis of uniform magnetite nanocrystals with high-quality properties for biomedical applications. *Journal of Materials Chemistry* **2012,** 22, (39), 21065-21075.
7. Lin, C.-A. J.; Sperling, R. A.; Li, J. K.; Yang, T.-Y.; Li, P.-Y.; Zanella, M.; Chang, W. H.; Parak, W. J., Design of an Amphiphilic Polymer for Nanoparticle Coating and Functionalization. *Small* **2008,** 4, (3), 334-341.
8. Caballero-Díaz, E.; Pfeiffer, C.; Kastl, L.; Rivera-Gil, P.; Simonet, B.; Valcárcel, M.; Jiménez-Lamana, J.; Laborda, F.; Parak, W. J., The Toxicity of Silver Nanoparticles Depends on Their Uptake by Cells and Thus on Their Surface Chemistry. *Part. Part. Syst. Charact.* **2013,** 30, (12), 1079-1085.
9. Anderson, R. E.; Chan, W. C. W., Systematic Investigation of Preparing Biocompatible, Single, and Small ZnS-Capped CdSe Quantum Dots with Amphiphilic Polymers. *ACS Nano* **2008,** 2, (7), 1341-1352.
10. Zhang, F.; Lees, E.; Amin, F.; Rivera_Gil, P.; Yang, F.; Mulvaney, P.; Parak, W. J., Polymer-Coated Nanoparticles: A Universal Tool for Biolabelling Experiments. *Small* **2011,** 7, 3113-3127.
11. Muir, B. W.; Moffat, B. A.; Harbour, P.; Coia, G.; Zhen, G. L.; Waddington, L.; Scoble, J.; Krah, D.; Thang, S. H.; Chong, Y. K.; Mulvaney, P.; Hartley, P., Combinatorial Discovery of Novel Amphiphilic Polymers for the Phase Transfer of Magnetic Nanoparticles. *Journal Of Physical Chemistry C* **2009,** 113, (38), 16615-16624.
12. Nazarenus, M.; Zhang, Q.; Soliman, M. G.; del Pino, P.; Pelaz, B.; Carregal_Romero, S.; Rejman, J.; Rothen-Ruthishauser, B.; Clift, M. J. D.; Zellner, R.; Nienhaus, G. U.; Delehanty, J. B.; Medintz, I. L.; Parak, W. J., In vitro Interaction of Colloidal Nanoparticles with Mammalian Cells: What Have We Learned Thus Far? *Beilstein Journal of Nanotechnology* **2014,** 5, 1477-1490.
13. Pelaz, B.; Charron, G.; Pfeiffer, C.; Zhao, Y. L.; de la Fuente, J. M.; Liang, X. J.; Parak, W. J.; del Pino, P., Interfacing Engineered Nanoparticles with Biological Systems: Anticipating Adverse NanoBio Interactions. *Small* **2013,** 9, (9-10), 1573-1584.
14. Dai, Q.; Walkey, C.; Chan, W. C., Polyethylene Glycol Backfilling Mitigates the Negative Impact of the Protein Corona on Nanoparticle Cell Targeting. *Angew Chem Int Ed Engl* **2014**.
15. Hühn, D.; Kantner, K.; Geidel, C.; Brandholt, S.; De Cock, I.; Soenen, S. J. H.; Rivera Gil, P.; Montenegro, J.-M.; Braeckmans, K.; Müllen, K.; Nienhaus , G. U.; Klapper, M.; Parak, W. J., Polymer-Coated Nanoparticles Interacting with Proteins and Cells: Focusing on the Sign of the Net Charge. *ACS Nano* **2013,** 7, (4), 3253-3263.
16. Lista, M.; Liu, D. Z.; Mulvaney, P., Phase Transfer of Noble Metal Nanoparticles to Organic Solvents. *Langmuir* **2014,** 30, (8), 1932-1938.
17. Yang, J.; Lee, J. Y.; Ying, J. Y., Phase transfer and its applications in nanotechnology. *Chemical Society Reviews* **2011,** 40, (3), 1672-1696.

18. Wang, X.; Xu, S.; Zhou, J.; Xu, W., A rapid phase transfer method for nanoparticles using alkylamine stabilizers. *Journal of Colloid and Interface Science* **2010,** 348, (1), 24-28.
19. Alkilany, A. M.; Yaseen, A. I. B.; Park, J.; Eller, J. R.; Murphy, C. J., Facile phase transfer of gold nanoparticles from aqueous solution to organic solvents with thiolated poly(ethylene glycol). *RSC Advances* **2014,** 4, (95), 52676-52679.
20. Goulet, P. J. G.; Bourret, G. R.; Lennox, R. B., Facile Phase Transfer of Large, Water-Soluble Metal Nanoparticles to Nonpolar Solvents. *Langmuir* **2012,** 28, (5), 2909-2913.
21. Alkilany, A. M.; Nagaria, P. K.; Hexel, C. R.; Shaw, T. J.; Murphy, C. J.; Wyatt, M. D., Cellular uptake and cytotoxicity of gold nanorods: molecular origin of cytotoxicity and surface effects. *Small* **2009,** 5, (6), 701-8.
22. Alkilany, A. M.; Thompson, L. B.; Boulos, S. P.; Sisco, P. N.; Murphy, C. J., Gold Nanorods: Their Potential for Photothermal Therapeutics and Drug Delivery, Tempered by the Complexity of their Biological Interactions. *Advanced Drug Delivery Reviews* **2012,** 64, (2), 190-199.
23. Wijaya, A.; Hamad-Schifferli, K., Ligand Customization and DNA Functionalization of Gold Nanorods via Round-Trip Phase Transfer Ligand Exchange. *Langmuir* **2008,** 24, (18), 9966-9969.
24. Huschka, R.; Zuloaga, J.; Knight, M. W.; Brown, L. V.; Nordlander, P.; Halas, N. J., Light-Induced Release of DNA from Gold Nanoparticles: Nanoshells and Nanorods. *Journal Of The American Chemical Society* **2011,** 133, (31), 12247-12255.
25. Kinnear, C.; Dietsch, H.; Clift, M. J. D.; Endes, C.; Rothen-Rutishauser, B.; Petri-Fink, A., Gold Nanorods: Controlling Their Surface Chemistry and Complete Detoxification by a Two-Step Place Exchange. *Angewandte Chemie-International Edition* **2013,** 52, (7), 1934-1938.
26. Liu, M.; Law, W.-C.; Kopwitthaya, A.; Liu, X.; Swihart, M. T.; Prasad, P. N., Exploring the amphiphilicity of PEGylated gold nanorods: mechanical phase transfer and self-assembly. *Chemical Communications* **2013,** 49, (81), 9350-9352.
27. Sekiguchi, S.; Niikura, K.; Matsuo, Y.; Ijiro, K., Hydrophilic Gold Nanoparticles Adaptable for Hydrophobic Solvents. *Langmuir* **2012,** 28, (13), 5503-5507.
28. Yang, J.; Lee, J. Y.; Deivaraj, T. C.; Too, H.-P., A highly efficient phase transfer method for preparing alkylamine-stabilized Ru, Pt, and Au nanoparticles. *Journal of Colloid and Interface Science* **2004,** 277, (1), 95-99.
29. Ye, X.; Zheng, C.; Chen, J.; Gao, Y.; Murray, C. B., Using Binary Surfactant Mixtures To Simultaneously Improve the Dimensional Tunability and Monodispersity in the Seeded Growth of Gold Nanorods. *Nano Letters* **2013,** 13, (2), 765-771.
30. Li, W. H.; Zamani, R.; Gil, P. R.; Pelaz, B.; Ibanez, M.; Cadavid, D.; Shavel, A.; Alvarez-Puebla, R. A.; Parak, W. J.; Arbiol, J.; Cabot, A., CuTe Nanocrystals: Shape and Size Control, Plasmonic Properties, and Use as SERS Probes and Photothermal Agents. *Journal Of The American Chemical Society* **2013,** 135, (19), 7098-7101.
31. Pellegrino, T.; Manna, L.; Kudera, S.; Liedl, T.; Koktysh, D.; Rogach, A. L.; Keller, S.; Rädler, J.; Natile, G.; Parak, W. J., Hydrophobic Nanocrystals Coated with an Amphiphilic Polymer Shell: A General Route to Water Soluble Nanocrystals. *Nano Letters* **2004,** 4, (4), 703-707.
32. Fernández-Argüelles, M. T.; Yakovlev, A.; Sperling, R. A.; Luccardini, C.; Gaillard, S.; Medel, A. S.; Mallet, J.-M.; Brochon, J.-C.; Feltz, A.; Oheim, M.; Parak, W. J., Synthesis and Characterization of Polymer-Coated Quantum Dots with Integrated Acceptor Dyes as FRET-based Nanoprobes. *Nano Letters* **2007,** 7, (9), 2613-2617.

33. Karakoti, A. S.; Das, S.; Thevuthasan, S.; Seal, S., PEGylated inorganic nanoparticles. *Angew Chem Int Ed Engl* **2011,** 50, (9), 1980-94.
34. Na, H. B.; Lee, I. S.; Seo, H.; Park, Y. I.; Lee, J. H.; Kim, S.-W.; Hyeon, T., Versatile PEG-derivatized phosphine oxide ligands for water-dispersible metal oxide nanocrystals. *Chemical Communications* **2007**, (48), 5167-5169.
35. Luo, M.; Shen, C.; Feltis, B. N.; Martin, L. L.; Hughes, A. E.; Wright, P. F. A.; Turney, T. W., Reducing ZnO nanoparticle cytotoxicity by surface modification. *Nanoscale* **2014,** 6, (11), 5791-5798.
36. Lu, C.; Bhatt, L. R.; Jun, H. Y.; Park, S. H.; Chai, K. Y., Carboxyl-polyethylene glycol-phosphoric acid: a ligand for highly stabilized iron oxide nanoparticles. *Journal of Materials Chemistry* **2012,** 22, (37), 19806-19811.
37. Wegner, K. D.; Morgner, F.; Oh, E.; Goswami, R.; Susumu, K.; Stewart, M. H.; Medintz, I. L.; Hildebrandt, N., Three-Dimensional Solution-Phase Förster Resonance Energy Transfer Analysis of Nanomolar Quantum Dot Bioconjugates with Subnanometer Resolution. *Chemistry of Materials* **2014,** 26, (14), 4299-4312.
38. Zhou, D.; Lin, M.; Chen, Z.; Sun, H.; Zhang, H.; Sun, H.; Yang, B., Simple Synthesis of Highly Luminescent Water-Soluble CdTe Quantum Dots with Controllable Surface Functionality. *Chemistry of Materials* **2011,** 23, (21), 4857-4862.
39. Ali, Z.; Abbasi, A. Z.; Zhang, F.; Arosio, P.; Lascialfari, A.; Casula, M. F.; Wenk, A.; Kreyling, W.; Plapper, R.; Seidel, M.; Niessner, R.; Knoll, J.; Seubert, A.; Parak, W. J., Multifunctional Nanoparticles for Dual Imaging. *Analytical Chemistry* **2011,** 83, (8), 2877-2882.
40. Roberts, M. J. B., M.D.; Harris, J.M., Chemistry for peptide and protein PEGylation. *Advanced Drug Delivery Reviews* **2002,** 54, 459 - 476.
41. Conde, J.; Ambrosone, A.; Sanz, V.; Hernandez, Y.; Marchesano, V.; Tian, F. R.; Child, H.; Berry, C. C.; Ibarra, M. R.; Baptista, P. V.; Tortiglione, C.; de la Fuente, J. M., Design of Multifunctional Gold Nanoparticles for In Vitro and In Vivo Gene Silencing. *Acs Nano* **2012,** 6, (9), 8316-8324.
42. Bartczak, D.; Muskens, O. L.; Sanchez-Elsner, T.; Kanaras, A. G.; Millar, T. M., Manipulation of in Vitro Angiogenesis Using Peptide-Coated Gold Nanoparticles. *ACS Nano* **2013,** 7, (6), 5628-5636.
43. del_Pino, P.; Pelaz, B.; Zhang, Q.; Maffre, P.; Nienhaus, G. U.; Parak, W. J., Protein corona formation around nanoparticles-from the past to the future. *Materials Horizons* **2014,** 1, 301-313.
44. Casals, E.; Pfaller, T.; Duschl, A.; Oostingh, G. J.; Puntes, V. F., Time Evolution of the Nanoparticle Protein Corona. *ACS Nano* **2010,** 4, (7), 3623-3632.
45. Tenzer, S.; Docter, D.; Kuharev, J.; Musyanovych, A.; Fetz, V.; Hecht, R.; Schlenk, F.; Fischer, D.; Kiouptsi, K.; Christoph Reinhardt; Landfester, K.; Schild, H.; Maskos, M.; Knauer, S. K.; Stauber, R. H., Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. *Nature Nanotechnology* **2013,** 8, (10), 772–781.
46. Maiorano, G.; Sabella, S.; Sorce, B.; Brunetti, V.; Malvindi, M. A.; Cingolani, R.; Pompa, P. P., Effects of Cell Culture Media on the Dynamic Formation of Protein-Nanoparticle Complexes and Influence on the Cellular Response. *ACS Nano* **2010,** 4, (12), 7481-7491.
47. Lee, S.; Anderson, L. J. E.; Payne, C. M.; Hafner, J. H., Structural Transition in the Surfactant Layer that Surrounds Gold Nanorods as Observed by Analytical Surface-Enhanced Raman Spectroscopy. *Langmuir* **2011,** 27, (24), 14748-14756.

48. Soenen, S. J.; Manshian, B.; Montenegro, J. M.; Amin, F.; Meermann, B.; Thiron, T.; Cornelissen, M.; Vanhaecke, F.; Doak, S.; Parak, W. J.; De Smedt, S.; Braeckmans, K., Cytotoxic Effects of Gold Nanoparticles: A Multiparametric Study. *ACS Nano* **2012,** 6, (7), 5767-5783.

TOC