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Ultra-stable and Biofunctionalizable Gold Nanoparticles

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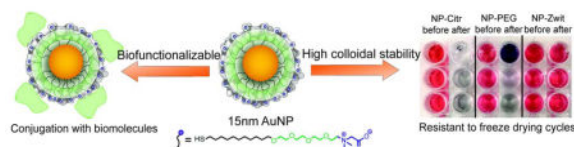
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Abstract

Gold nanoparticles provide an excellent platform for biological and material applications due to their unique physical and chemical properties. However, decreased colloidal stability and formation of irreversible aggregates while freeze-drying nanomaterials, limits their use in real world applications. Here, we report a new generation of surface ligands based on a combination of short oligo (ethylene glycol) chain and zwitterions capable of providing non-fouling characteristics, while maintaining colloidal stability and functionalization capabilities.

Additionally, conjugation of these gold nanoparticles with avidin can help developing a universal toolkit for further functionalization of nanomaterials.

Graphical abstract



Keywords

Stability; Gold nanoparticles; UV; DLS; ion strength; freeze-drying; surface plasmon resonance; biofluids

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‡Author Contributions

A.G. and D.F.M. contributed equally to this work.

Notes

The authors declare no competing financial interests.

Supporting Information.

Nanoparticle synthesis, characterization, and additional experimental details and figures are described in the supporting information.

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INTRODUCTION

Gold nanomaterials have been used in numerous applications, from the creation of sensors and catalysts,^{1,2,3} to their applications as drug delivery vehicles and diagnostics agents.^{4,5,6} Among the different sizes of gold nanoparticles (AuNPs), medium/large size (10 nm) gold nanoparticles have been extensively investigated due to their unique physical properties.^{7,8} For example, the well-defined surface plasmon resonance (SPR) of these particles has been employed for sensing, where a variety of sensors have been developed by following changes of the SPR during the sensing event.^{9,10,11} By the use of this colorimetric strategy, not only the detection of small molecules and ions is possible, but also more complex systems such as proteins, cells, and by extension diseases can be detected.^{12,13,14,15}

The sensitivity and specificity of these AuNP systems can be tuned by functionalizing the particle surface with different ligands (e.g. protein receptors).¹⁶ This facile change of the AuNP surface identity has also offered opportunities for the utilization of these nanomaterials in biological applications. The use of proteins as one of the surface ligands, for example, has allowed the development of targeted carriers to deliver drugs in specific locations.^{17,18,19} Likewise, the conjugation of AuNPs with DNA strands has permitted the utilization of AuNPs for imaging and therapy, where detection or regulation of genes has been achieved.²⁰

Despite of the potential of AuNPs to serve as sensor and delivery platforms, the use of these material in real world applications is limited by their lack of long-term colloidal stability, in particular the formation of irreversible aggregates when they are subjected to physical and chemical changes (e.g. contact with biofluids, freeze-drying, and ion strength gradients). Particle stability is an important prerequisite for the use of AuNPs for in vitro and in vivo applications, and as such this stability issue complicates the use of AuNPs in biological environments.^{21,22} In addition, this lack of stability limits the industrial implementation of these systems, given that the low stability complicates various steps of the manufacturing and shipping processes.²³ To counter the challenge of instability, several approaches have been investigated.^{24,25,26} The most successful method to date is the use of non-ionic poly(ethylene glycol) (PEG) to functionalize the AuNPs, a technique that is capable of conferring some degree of stability to AuNPs.^{27,28} However, the absence of reactive centers in this molecule combined with its amphiphilic nature hinders the systematic functionalization of PEG stabilized AuNPs.^{29,30} For example, the presence of PEG allows the internalization of hydrophobic functionalities masking their effects, while the amphiphilic nature of PEG alters the bioactivity of proteins.^{31,32} In addition, a report presents evidence of the recognition of PEG structures by the immune system, triggering a rapid elimination from the body, thus limiting its use.³³

In our previous studies we have shown that zwitterionic ligands provided very tractable small (2 nm core) AuNPs.³⁴ Here we present the use of zwitterionic ligand to provide high colloidal stability to medium and large AuNPs. Through appropriate carboxybetaine ligand design we have developed particles with 'plug and play' streptavidin connections for ready conjugation. Crucially, AuNPs decorated with these ligands can be stored as powders, exhibiting resistance to freeze-drying as well as changes in ionic strength while still

maintaining functionalization capabilities. These conjugates do not lose their activity and stability after freeze-drying, facilitating the functionalization of nanomaterials with other proteins or mixture of molecules. As such, this NP coverage confers nanomaterials the capability of being used as building blocks, allowing not only an easy and fast functionalization, but also enabling the development of new technologies for therapeutic applications that cannot be achieved with unstable nanoparticles.

RESULTS AND DISCUSSION

Fabrication and characterization of different size AuNPs

The chemical design of the NP ligand comprises the combination of a short oligo (ethylene glycol) (OEG) chain and a carboxybetaine zwitterionic headgroup (Figure 1a). In previous studies, we demonstrated that by the use of a similar design we were capable of reducing protein adsorption over small nanoparticles (<10 nm),³⁴ as well as confer increased blood circulation times comparative to PEG.³⁵ That made us aware of the possibilities of increasing colloidal stability by the use of this approach. However, chemical diversity was limited to pre-functionalization synthesis, as sulfobetaines do not offer readily activatable chemical groups. To overcome this difficulty in functionalization, in our current design we employed a carboxybetaine group,^{36,37,38} a moiety that allowed us to vary the end group at will after the final AuNP synthesis (ligand synthesis, AuNP functionalization and characterization are described in the supporting information, Figures S1–S5). Incorporation of carboxybetaines as zwitterionic headgroups conferred high colloidal stability to the AuNPs. In particular, it allowed us to design “plug and play” systems that could prevent non-specific protein adsorption and remain biofunctionalizable at the same time. We demonstrated the utility of these particles using three different gold NP core sizes, 15 nm, 25 nm, and 35 nm diameter, given the significant amount of applications that have been developed for particles of this size. It should also be pointed out that 15 nm as the current industrial AuNP standard. The functionalization of the 15 nm core with the zwitterionic ligand did not affect the core size of the AuNPs and increase in the hydrodynamic size is accounted to the ligand attachment (Figure 1b, c)

Stability studies of zwitterionic-AuNPs

We first tested the stability of AuNPs functionalized with our ligand (AuNP-Zwit) comparative to citrate-capped AuNPs (AuNP-Citr) and thiolated-PEG functionalized AuNPs (AuNP-PEG), highly used in biological applications. To this end, we varied the ionic strength and observed changes in color (SPR peak), indicative of AuNP aggregation.^{39,40,41} As expected, citrate capped AuNPs aggregated with increasing ionic strength, with a change in the original SPR peak observed as the salt concentration increased (Figure 2a, 2b, 2c and 2d). AuNP-PEG also showed signs of aggregation at high salt concentrations. We only observed a decrease in stability at extreme salt concentrations for the largest gold core (35 nm, Figure S6). On the other hand and as observed in Figure 2c and 2d, our ligand was capable of conferring stability to AuNPs even at extreme ion concentrations (5M), where no apparent changes in the color were observed. Additionally, zwitterionic NPs were stable even after incubation for 24 hours at high salt concentration (up to 5mM), which is

evidenced by the UV spectrum in Figure S6 d). This further corroborates long term storage stability conferred by our zwitterionic ligands.

One of the principal barriers for the industrial and general use of AuNPs is their poor capability of enduring freeze-drying cycles.²³ This is one of the major reasons of why most of the commercially available AuNPs are sold as solutions. In addition, many reports have shown that when AuNPs are subjected to lyophilization,⁴² larger AuNPs agglomerates are formed, and these aggregates cannot be dispersed again in solution.⁴³ Variations in the stabilizing agent helps in controlling shape and the size of the final structures, though complete stability was achievable only in few cases.^{44,45} For example, one report showed that 15 nm AuNPs are capable of resisting freeze-drying cycles if PEG structures were used as the surface ligands. However, as we described before, this technique does not facilitate the post-functionalization of AuNPs with ligands of interest, limiting its applicability.

Effective stabilization of NPs often requires bulky PEG ligands (~5Kd) which alter their mobility.⁴⁶ We were interested in observing if AuNPs decorated with our ligand were capable of enduring this stressing process. For this purpose we subjected the AuNP-Zwit to freeze-drying, and records of the UV spectrum were taken before and after the process. As observed in Figure 2e–f (and Figure S7), the initial UV spectrum is essentially the same to the one obtained for AuNP-Zwit redispersed in water after lyophilization. This signal recovery was observed even after repeating the freeze-drying cycle 10 consecutive times, evidencing the degree of colloidal stability that the zwitterionic ligand can confer to the different AuNP sizes. On the contrary, AuNP-PEG and citrate-capped AuNPs could not be redispersed after the first lyophilization cycle (Figure S8).

Understanding the association of NPs with proteins plays an important role in biosensing and biomedical applications. Avoiding interactions such as formation of a protein corona is important for designing ‘stealthy’ particles for delivery applications. The undesirable adsorption of proteins on NP surface not only reduces its targeting capabilities but it also promotes their elimination from the body. We tested stability of our AuNPs in biological fluids (human serum) by dynamic light scattering (DLS), as this is critical a requirement for their use in applications in vitro and in vivo.^{47,48} As depicted in the DLS profiles (Figure 3), no aggregates were observed for AuNP-Zwit while AuNP-Citr and AuNP-PEG formed larger aggregates, evidencing the enhanced stability conferred by the zwitterionic ligand.

Bio-functionalization of AuNPs

After the colloidal stability of our system was tested, we then proceeded to functionalize the surface of the AuNPs with biomacromolecules according to our initial design (inclusion of the carboxylic group in the zwitterionic pair). For this experiment we selected the 15 nm gold core as this is size is the one that be found from commercial sources. Since our intention was to create a ‘plug-and-use’ system of AuNPs, we covalently linked AuNP-Zwit to avidin by the use of EDC/NHS coupling.^{49,50} Each NP could bind to 9 avidin molecules as determined using biotin-FITC binding experiments (described in Supporting Information).⁵¹ The purpose of using avidin was to further allow the system to form conjugates with any biotin-functionalized biomacromolecule in an easy and fast way (Figure 4a).⁵² To assess if this arrangement was possible, we mixed biotin-HRP (horseradish

peroxidase) with the avidin-functionalized AuNP-Zwit. After removing the free HRP through the use of numerous centrifugation/redispersion cycles, we tested if the conjugation was successful by introducing ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) to the solution and observing if coloration was observed (indicating the presence of HRP). As observed in Figure 4b, a significant change in color was observed comparative to the controls, evidencing the formation of the conjugates. We then determined if the AuNP-Zwit/avidin conjugates were capable of enduring freeze-drying cycles. As such, we lyophilized the AuNP-Zwit/avidin conjugates and analyzed if the avidin maintained its capability of binding to biotin-HRP. As depicted in figure 4b, this was the case and the AuNP-HRP conjugates were still formed. Furthermore, the kinetics of substrate consumption was similar to the one observed before the lyophilization, indicating no lost in the structural conformation required for the avidin-biotin recognition (Figure 4c). It is important to note that the formulation of these conjugates was done by simply mixing AuNPs-Zwit/avidin with biotin-HRP, followed by a fast cleaning process with centrifugation. This convenient conjugation procedure puts this approach closer to the idea of 'disperse and use' commonly required for the majority of therapeutic formulations.

CONCLUSION

In summary, we have introduced a new nanoparticle coverage specifically designed to provide enhanced stability to AuNPs while still offering functionalization capabilities. AuNPs functionalized with this ligand are stable in biological fluids, are able to endure strong changes in ionic strength, and can be redispersed after freeze-drying cycles. In addition, these AuNPs can be functionalized with biomacromolecules, and by the specific use of avidin, any biotin-functionalized macromolecule (from the large catalog that is commercially available) can be used to confer the AuNPs the desired biological properties. Finally these AuNPs-avidin conjugates can be lyophilized without affecting their activity, which allows their transport as powders for future formulations. As such, this approach will allow the convenient development of therapeutic formulations, and will facilitate the introduction of these technologies into the industrial scale.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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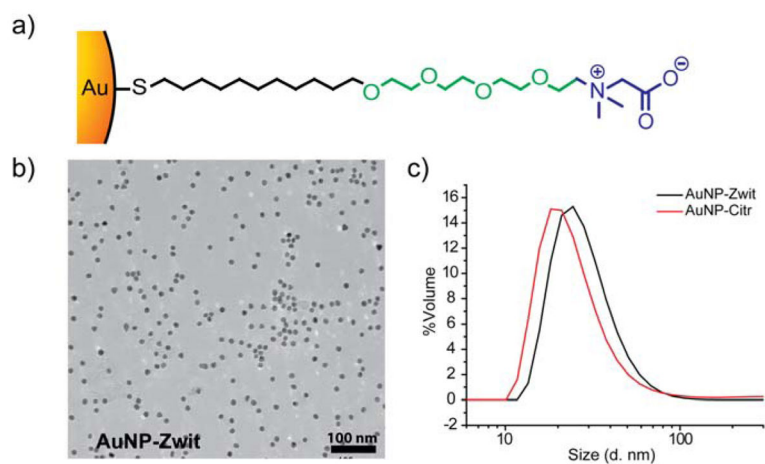


Figure 1.

a) Chemical structure of the AuNP ligand, composed of an oligo (ethylene glycol) chain followed by a carboxybetaine zwitterionic group. b) TEM of the 15 nm AuNPs after functionalization with the engineered ligand (AuNP-Zwit). c) DLS profiles of AuNP-Zwit and AuNP-Citr (without ligand functionalization) in phosphates buffer (PB).

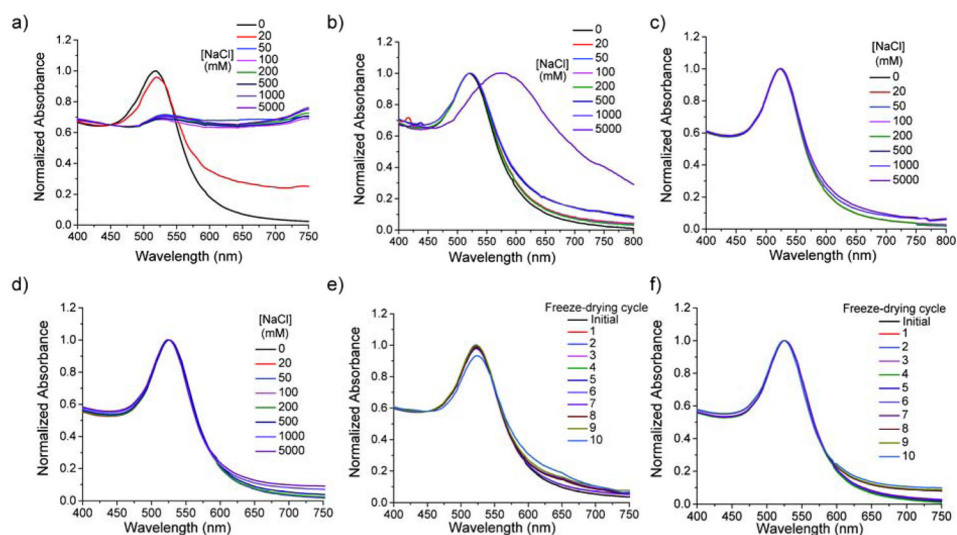


Figure 2.

a) UV spectra of AuNP-Citr, b) 15 nm AuNP-PEG, c) 15 nm AuNP-Zwit and d) 25 nm AuNP-Zwit at different ion strengths, evidencing the enhanced stability conferred by the zwitterionic ligand. e) UV spectrum of 15 nm and f) 25 nm AuNP-Zwit, after successive cycles of freeze-drying.

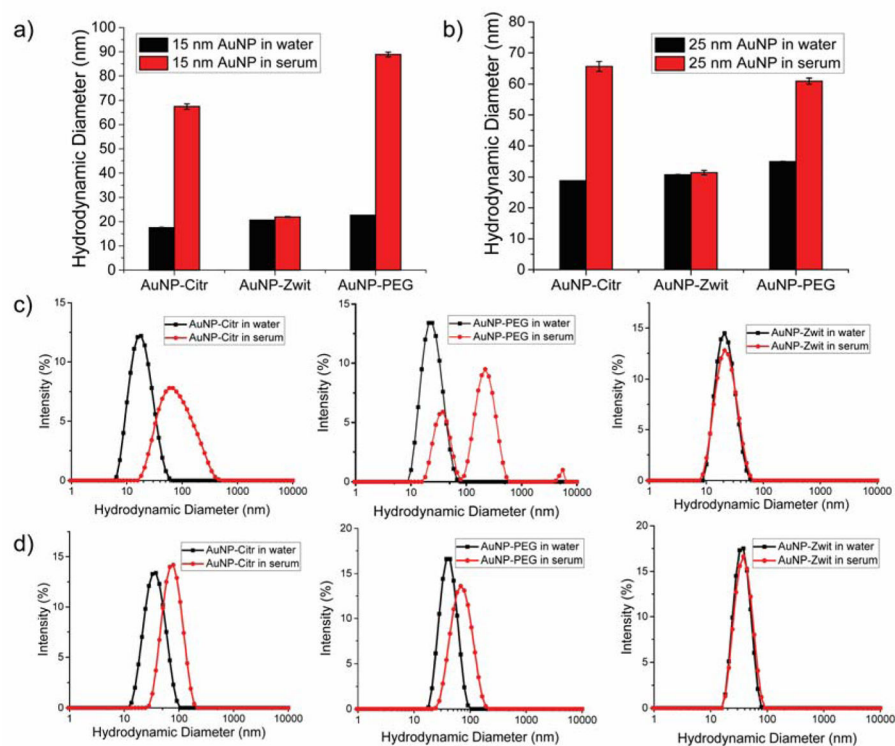


Figure 3. Comparison of NP size after 24 hr incubation in serum a) 15 nm AuNPs b) 25 nm AuNPs. Particle size distribution for NPs in the presence and absence of serum proteins (50%) evidencing the stability of AuNP-Zwit c) DLS profiles of 15 nm AuNP-Citr, AuNP-PEG and AuNP-Zwit and d) DLS profiles of 25 nm AuNP-Citr, AuNP-PEG and AuNP-Zwit respectively.

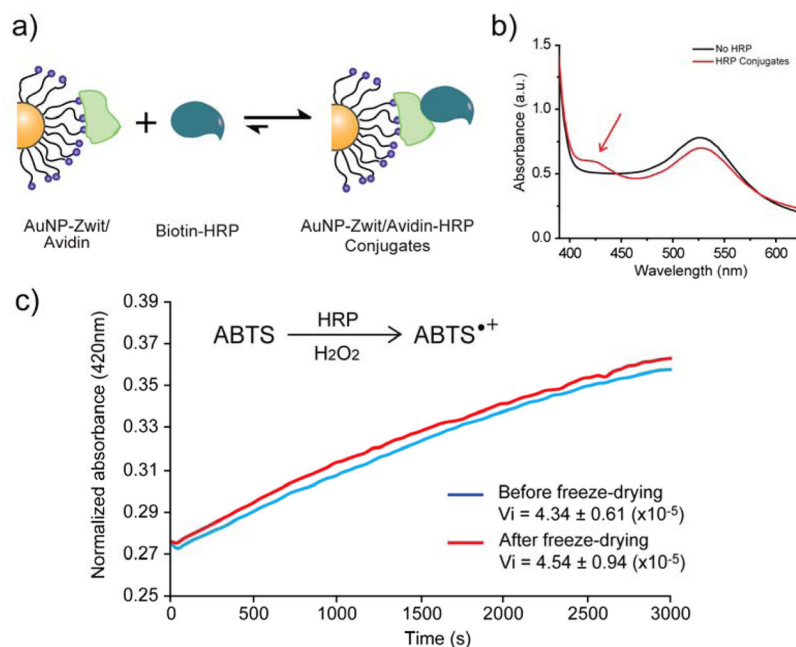


Figure 4.

a) Scheme of the conjugation of AuNP-Zwit/avidin with biotin-functionalized HRP. b) UV spectrum of AuNPs alone and conjugated with HRP after the addition of ABTS (substrate). c) Normalized substrate transformation by the AuNP-Zwit/avidin-HRP conjugates before and after freeze-drying. As evidenced by the initial rates of reaction (V_i), the AuNP-Zwit/avidin conjugates are capable of enduring freeze-drying stress without losing activity.