



Stability and biocompatibility of photothermal gold nanorods after lyophilization and sterilization



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ABSTRACT

Suspensions in phosphate buffered saline (PBS) of gold nanorods stabilized with cetyltrimethyl ammonium chloride (CTABr), polystyrenesulfonate (PSS) and methyl-polyethyleneglycol-thiol (m-PEG-SH) have been prepared and the evolution of their colloidal stability and plasmonic response over time has been evaluated. Their performance after lyophilization, alcoholic sterilization and resuspension has also been characterized. Sub-cytotoxic doses on HeLa cells were calculated for the three surface functionalizations used. Their heating efficiency at different exposure times was also evaluated after being irradiated with near infrared light. The best results were obtained for m-PEG-SH stabilized rods, which were not only stable, sterilizable and lyophilizable, but also biocompatible at all doses tested, showing potential as a stable, re-suspendible and biocompatible hyperthermic agent.

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1. Introduction

Nanoparticles exhibit physical properties depending on their size whereas constant properties are expected for bulk materials whatever their size. Quantum confinement in semiconductors, superparamagnetism in magnetic nanoparticles, catalytic activity and localized surface plasmon resonance (SPR) in noble metal nanoparticles are clear examples of physical phenomena which occur only at the nanoscale. Also, the presence or absence of specific metastable crystalline phases in ceramics, melting-point depression and semiconductivity in metal nanoparticles are physical phenomena related to the nanoscale. Many industrial applications of nanoparticles are based on those physical properties; however, those often vanish when processes leading to changes in size or shape, or to agglomeration or aggregation (irreversible agglomeration) occur. As a consequence, many strategies have been developed to stabilize nanomaterials and their suspensions.

Nanoparticle dispersions can agglomerate due to attractive forces such as van der Waals, electrical double-layer interactions (Coulombic interactions), hydrogen bonding, and by hydrophobic and solvation phenomena [1]. The colloidal stability of those dispersions is achieved by steric hindrance usually by grafting or adsorbing neutral polymers with long alkyl chains (i.e., PEG, PVP, PAAm, poly(oxazolines), etc.) on the surface of the nanoparticles which prevent them from approaching. Also electrostatic repulsion is widely used to stabilize a colloid by means of surface functionalization in such a way that at the working pH the colloid shows a zeta potential (generally above ± 30 mV) in the interfacial double-layer high enough to achieve electrostatic repulsion between the colloid-forming nanoparticles. This electrostatic surface functionalization is commonly carried out by using ionic surfactants (i.e., cetyltrimethyl ammonium chloride (CTABr), dimethylammonium bromide (DMABr), sodium dodecyl sulfate (SDS), etc.) and synthetic (i.e., poly(ethyleneimine) (PEI), poly(sodium 4-styrenesulfonate) (PSS), poly(allylamine hydrochloride) (PAH), poly(diallyldimethylammonium chloride) (PDDA), etc.) or organic polymers (i.e., polysaccharides such as starch, chitosan, dextran; polypeptides, cationic phospholipids, and so on) which can be adsorbed on the surface of the nanoparticles or directly attached by using electrostatic or covalent interactions. Functional groups are also used to render electrostatic repulsion between the nanoparticles by direct grafting on their surfaces (i.e., amino-silane grafting on the surface of silica nanoparticles). As already mentioned, colloidal stability is generally

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needed because physical properties are size dependent but becomes even more critical when biomedical applications are envisaged. The interaction between nanoparticles and biological environments defines any potential application in the field of nanomedicine and a precise control of the nanoparticle-surface characteristics is needed for a suitable design of that interface.

Usually, engineered nanoparticles are spherical in shape because spheres have the smallest surface area per unit volume of any geometry and therefore, provide an efficient way to minimize their surface free energy [2]. However, other shapes are also possible and some physical properties (e.g., optical) are dependent on the nanoparticle geometry. For example, due to their anisotropy, gold nanorods (NRs), which are the subject of this work, display two SPR peaks corresponding to the transverse and longitudinal plasmon bands. NRs are interesting photonic nanomaterials used in catalysis [3,4] and in different biomedical applications including photothermal therapy against tumoral cells [5] or bacteria [6] and also in medical imaging by means of optical coherence tomography [7] or luminescence [8], and so on. Biosensing [9–11], optical labeling [12,13] and optical nanocomposites [14] are other examples of the various applications of those anisotropic noble metal nanoparticles.

In biomedical applications, where the interaction of the nanoparticle surface with the different biomolecules defines their final fate, PEG and PEG-containing copolymers stand out as superior polymers used to avoid protein (opsonins) adsorption [15], by creating hydrated barriers, and consequent recognition and clearance by the mononuclear phagocyte system, prolonging their circulation life span when nanoparticle intravenous administration is envisaged [16]. In general, to avoid protein adsorption, neutral or zwitterionic and hydrophilic polymers are preferable compared to ionic and hydrophobic ones [17].

Also aqueous solutions are preferable to prepare biocompatible NRs dispersions over organic-solvent based routes due to the complexity of the phase transfer of the later. Usually, for organic dispersions a labile ligand capping the surface of the nanoparticles produced in a non-polar solvent is replaced by the desired aqueous biofunctional ligand [18]. Monofunctional or bifunctional PEGs can be covalently attached to the surface of NRs, generally by means of thiol or amino groups, and under these conditions yield dispersions that are stable under physiological conditions, avoiding undesired desorption or displacement by simple electrostatic or hydrophobic interactions. A strong chemical bond between nitrogen present in the amino groups and the gold orbitals via their lone pairs is responsible for this strong attachment [19] and a semi-covalent bond between gold and sulfur present in the thiol is used when thiolated PEGs are grafted to the surface of the nanoparticles [20,21]. The key issue is to achieve colloidal stability under physiological conditions in a media with moderate to high ionic strength (100 mM KCl) and also at the physiological pH.

The synthesis of Au nanorods often involves CTABr as a surfactant. However, different examples in the literature reveal that the apparent cytotoxicity is caused by free CTABr in solution [22] and describe how CTABr-coated nanorods aggregate under physiological conditions and also the fact that this surfactant might be cytotoxic due to its ability to degrade biomembranes and peptides *in vitro* and *in vivo* [23,24]. Therefore, different strategies have been developed to avoid their SPR red-shift or quenching upon aggregation and to promote biocompatibility. Most potential medical applications of nanorods would require both, particle and suspension stability under short to medium term (days to weeks) storage. Particle stability refers to maintaining the plasmonic properties of the particles until they are used. This, for Au NRs implies maintaining their shape, avoiding the evolution toward lower aspect ratio geometries. In this work, plasmonic properties under physiological conditions as well as cytocompatibility are

addressed when using surfactant-capped NRs compared to polystyrenesulfonate (PSS) and PEG (m-PEG-SH) coated NRs. The stability and ability to reconstitute after freeze-drying and sterilization are also evaluated for the three different surface-functionalized NRs. PSS is widely used as electrostatic stabilizer of NRs by means of its negative charge given by the deprotonated sulfonyl groups [25].

The goal of this work was to study the colloidal stability of different surface functionalized gold nanorods over time as well as their ability to be freeze-dried and re-suspended together with their stability after sterilization.

2. Materials and methods

2.1. Gold nanorods synthesis, surface modification and characterization

NRs were synthesized in aqueous solution by the silver ion-assisted seed-mediated protocol described by Pastoriza-Santos et al. [25]. In brief, CTABr and silver seeds are used to achieve an anisotropic growth of the rods due to their preferential adsorption on the (1 1 0) crystallographic facets of gold [23,26]. PSS was electrostatically attached to the surface of the positively charged NRs-CTABr by using the same protocol [25]. Methyl-polyethyleneglycol-thiol (m-PEG-SH) was covalently attached to the surface of the NRs-CTABr following the experimental protocol described by Maltzahn et al. [24]. In this case, a dispersion of NRs containing CTABr was centrifuged, decanted, and re-suspended in deionized water to remove excess of CTABr. Subsequently, m-PEG-SH was added to this dispersion and the unbound PEG was removed by dialysis. The resulting NRs were labeled as NRs-mPEG.

Dispersions of NRs-mPEG, NRs-CTABr or NRs-PSS were incubated in PBS for different periods of time. At regular intervals, samples were spectrophotometrically analyzed (UV-vis-NIR spectrophotometer, Jasco V670, Tokyo, Japan) for potential plasmon resonance peak shifts or emission quenching, which would indicate particle agglomeration. Transmission Electron Microscopy (TEM, FEI™ Tecnai G2 F30 Microscope, Hillsboro, USA) was used to examine the morphology of the synthesized nanoparticles. Zeta potential of the corresponding NRs dispersions was obtained by using dynamic light scattering (DLS) measurements (90Plus Particle Size Analyzer by Brookhaven Instruments Corp., Holtsville, USA). Fourier transform infra-red (FTIR) spectroscopy of the different particles was performed with a Bruker Vertex 70 FTIR, Ettlingen, Germany spectrometer equipped with a DTGS detector and a Golden Gate diamond ATR accessory. Spectra were recorded by averaging 40 scans in the 4000–600 cm^{-1} wave number range at a resolution of 4 cm^{-1} . Data evaluation was carried out by using the OPUS software from Bruker Optics, Inc. The ability of being reconstituted in PBS after freeze-drying and sterilization was also evaluated by UV-vis-NIR spectroscopy. Alcoholic sterilization was carried out by using a closed chamber in which ethanol vapor was introduced by means of a saturator.

2.2. Photothermal measurements

Colloidal PBS suspensions (4 mL, 2 mg/mL) of re-suspended NRs-PSS and NRs-mPEG (obtained after lyophilization) were directly exposed to continuous NIR light for 20 or 40 min. The IR Laser System consists in a 808 nm wavelength laser diode (6 × 8 mm spot size) (Optilas model MDL-III-808-2W, Changchun New Industries Optoelectronics Technology Co., Ltd., Changchun, China) and a power controller (Model PD300-3W, Ophir Laser Measurement Group, Logan, UT, USA), placed in a thermostatic chamber (Model Stuart SI60D, Fisher Scientific Afora, Madrid,

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