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Biofunctionalization of gold nanorods*

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Abstract: Gold nanorods (NRs) are promising nanomaterials for biotechnology innovations that include photoassisted drug delivery, gene therapy, noninvasive cancer detection, and ultrasensitive biodetection. Owing to their unique geometry, Au NRs exhibit surface plasmon (SP) modes in the near-infrared (IR) wavelength range—ideal for carrying out optical measurements in biological fluids and tissue. Because NR interactions highly depend on the chemical nature of their solvent-accessible interface, it is necessary to carry out specific postsynthetic chemical modifications of the Au surface to create NRs that are biocompatible and biofunctional. In this review, we discuss various NR surface chemistries that have successfully enabled the integration of Au NRs into biological environments, as well as current challenges in designing the biofunctional NR interface for in vivo applications.

Keywords: biosensing; biotechnology; metals; nanoparticles; nanorods; surface modification.

INTRODUCTION

Noble metal nanostructures made of Ag or Au exhibit unique electromagnetic properties and are expected to play a key role in advancing current optical tools for biological imaging [1–3], chemical sensing and detection [4–7], medical diagnostics [8–10], and biomedical therapy [11–13]. Nanostructures with engineered shapes behave like optical antennae by concentrating electromagnetic fields due to the excitation of surface plasmons (SPs). SP excitation is ultrasensitive to nanostructure geometry, and Au nanorods (NRs) exhibit this shape dependence beautifully, where the color of colloidal NR dispersions can be designed to span the visible spectrum based on varying NR aspect ratios [14]. Due to their anisotropic shape, Au NRs possess two distinct SP modes that oscillate in the transverse and longitudinal direction of the NR axis. Modulation of SP wavelength advantageously allows SP excitation to be tuned in the near-infrared (IR) wavelength range, which is ideal for optical imaging or spectroscopic analysis of biological tissues. Thus, Au NRs have been pursued for a number of biotechnology applications, from low-level protein sensing to noninvasive cancer surveillance.

A major challenge for the realization of nanomaterials in these sensing or biomedical applications stems from chemical modification of the inorganic surface. The solvent-accessible surface of Au NRs must be compatible for introduction into biological environments, where chemical interactions that take place at the NR interface can be difficult to characterize. NRs introduced into cells or tissue experience a crowded, dynamic local environment. Typically, as-synthesized Au NRs are stabilized by a strongly coordinated ligand shell of surfactant that passivates the Au surface. Because NR interactions are mostly governed by the chemical nature of their surfaces, a substantial need exists for postsynthetic chemical modification strategies that offer greater command over the biological/inorganic interface. In

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this review, we highlight various NR surface chemistries that enable integration of Au NRs into biological environments and biofluids, as shown in Fig. 1. Here, we discuss successful approaches for surface modification that have been demonstrated for Au NRs, where the resulting NRs are biocompatible, exhibit minimal cytotoxicity, and are biofunctional.

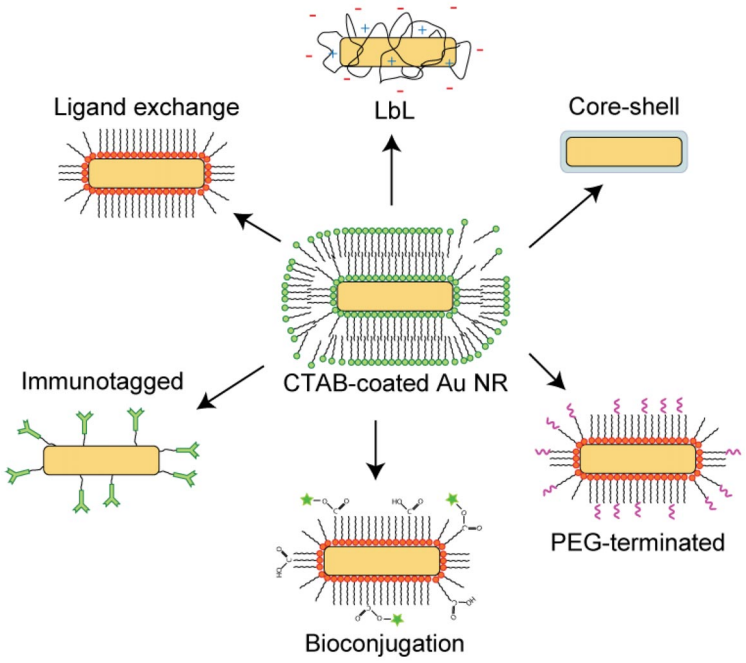

Fig. 1 Schematic of different strategies for the biofunctionalization of Au NRs, beginning with the as-synthesized NR coated with a bilayer of CTAB molecules.

NANOROD SYNTHESIS AND PROPERTIES

Synthesis of Au NRs

The most widely used protocol for synthesizing Au NRs is the seed-mediated, surfactant-directed method described by Jana et al., where $HAuCl_4$ is reduced in aqueous solutions of hexadecyltrimethylammonium bromide (CTAB) [15]. During the NR growth process, CTAB serves as a stabilizing surfactant that directs one-dimensional NR growth by protecting the (100) and (110) crystallographic Au facets of nucleated nanoparticles, promoting selective Au deposition at the (111) facets to form an elongated structure. This procedure yields crystalline NRs with diameters between 10–20 nm and variable lengths depending on the seeding conditions. The growth mechanism of Au NRs has been discussed in a number of excellent, in-depth articles [14,16,17] and thus will not be discussed in detail here. However, the nature of the CTAB–Au interface is highly relevant to our discussion of NR biofunctionalization. CTAB is known to form a bilayer structure around the as-made Au NRs, and the CTAB–Au interaction of the inner monolayer is largely electrostatic (rather than covalently bound). As a result, CTAB can be labile at the NR interface depending on solvent conditions, and desorption of CTAB from the NR surface has been observed to induce morphology changes and aggregation of the Au NRs [18]. Free-floating CTAB molecules have also been demonstrated to exhibit high cytotoxic effects [19]. Thus, a major challenge to utilizing Au NRs in practical biological applications is the removal or displacement of CTAB from the NR surface.

Surface plasmon properties

In contrast to spherical Au nanoparticles which possess a single dipolar SPR mode, Au NRs with these dimensions exhibit two distinct SP modes correlated to their anisotropic geometry (Fig. 2): the transverse SP mode at λ = 520 nm and the longitudinal SP mode at λ = 810 nm (for an NR with dimensions of 10 × 40 nm) [20,21]. It is well known that by increasing the NR aspect ratio, the longitudinal SPR band red-shifts into the near-IR to IR wavelength range, enabling tunable electromagnetic properties through careful control of the NR growth process. These SP properties are especially desirable for biomedical applications since optical readouts within the near-IR wavelength range encounter minimal background due to absorption or scattering from endogenous chromophores and water [3,10]. Au NRs have been impressively demonstrated as excellent optical tags for tissues, cells, and biomolecules given the large absorption cross-section of NRs at the longitudinal λ_{SP} [2,22].

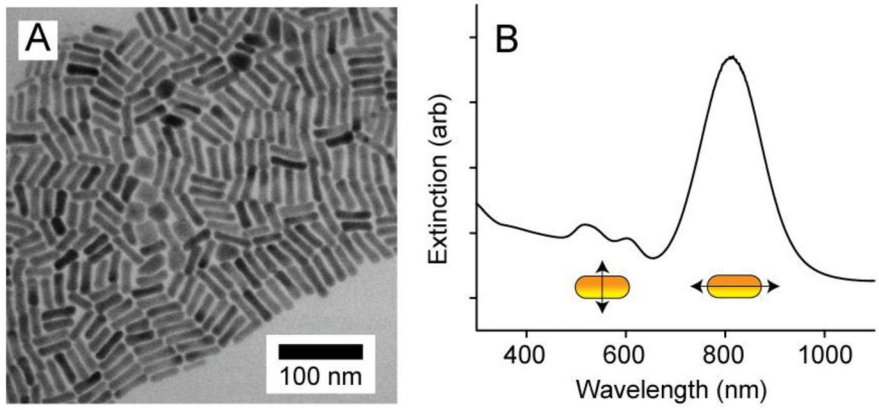

Fig. 2 Scanning transmission electron microscope image (A) and corresponding UV–vis (B) spectrum for a dispersion of Au NRs. The peak at λ_{SP} = 810 nm corresponds to the longitudinal SP mode that oscillates along the axis of the NR.

For SP-based biosensing, optical readouts have the potential to be sensitive to chemical fluctuations at the single-molecule level. The key concept underlying plasmonic sensing is that λ_{SP} and scattering intensity are dependent on the surrounding dielectric environment of the NR, limited to the local evanescent field at the Au surface. Particularly high sensitivities for molecular detection are enabled by sharp nanoscale features that produce large electrodynamic field enhancements at the metal surface, such as those presented at the NR tips. According to electromagnetic simulations, the bulk refractive index sensitivity of an SP band is linearly correlated with the wavelength of SP peak for particles of a specified composition [23]. Thus, compared to spherical Au nanoparticles which exhibit a SPR peak around 520 nm, Au NRs with a longitudinal mode between 750–900 nm possess a greater degree of optical sensitivity to changes in their local environment [21].

Photothermal properties

Au NRs display unique photothermal properties when excited at λ_{SP}. Au NRs convert incident photons into heat (up to 96 %) more effectively than other shaped nanostructures such as nanospheres and nanoshells [2,24]. Thus, considerable efforts are currently being directed toward Au NRs for in vivo photothermal treatment of cancer, where long-wavelength (650–900 nm) laser irradiation can effectively penetrate tissue. For example, Huang et al. demonstrated that Au NRs conjugated to anti-epidermal growth factor receptor (anti-EGFR) antibodies could selectively adhere to the surfaces of malignant

cancer cells over non-malignant cells; irradiation using a continuous-wavelength 800 nm laser at low power resulted in selective photothermal injury of malignant cells [1]. SP excitation of NRs is also able to induce local thermal melting of the Au surface, enabling photoactivation or deactivation of conjugated NR surfaces for various payload deliveries [13]. By functionalizing Au NRs possessing different aspect ratios with different biomolecular payloads, irradiation at the appropriate λ_{SP} can instigate selective Au NR melting and subsequent payload release.

GOLD-BINDING LIGANDS

The most readily available chemistry for postsynthetic surface modification of Au NRs is CTAB displacement with alkanethiol molecules that form strong Au–S covalent bonds at the NR surface. It is well known that long-chain alkanethiols self-assemble to form well-ordered, solidly packed molecular monolayers on Au and Ag surfaces [25,26]. Ligand exchange reactions in excess concentrations of these ligands are a facile and effective surface passivation strategy for Au NRs. Further, the solvent-accessible interface of the Au NR can be chemically tuned by thoughtfully choosing an alkanethiol functionalized with a given end group. In addition to tuning solvent miscibility [27,28], end groups can be chosen to impart high surface-charge densities to the NR [29] or to function as chemical handles for carrying out conjugation chemistry at the NR surface [18]. For example, 5'- and 3'-(alkanethiol)-capped oligonucleotides can be used to generate DNA-Au NR conjugates to control localized gene expression [30]. Upon cellular uptake, these DNA-Au NR conjugates can be used as photothermally activated payload carriers where high-intensity pulses of near-IR light destabilize the NR surface and serve as the trigger for DNA release. In this case, the strong Au–S linkage prevents DNA release, and hence gene expression, from occurring without irradiation at the appropriate λ_{SP}.

However, the strong nature of the CTAB–Au interaction may hinder complete displacement of the CTAB by thiol-functionalized molecules, especially by those that possess a low driving force for self-assembly at the Au surface (i.e., due to steric hinderance or a short hydrocarbon chain). At limiting alkanethiol concentrations, this can lead to unique geometric effects where CTAB displacement does not occur homogenously. Several groups have observed that ligand exchange under these conditions is characterized by selective displacement of CTAB at the (100)-terminated NR tips, where NR curvature dictates a lower density of CTAB coverage on the Au surface and greater solvent accessibility into the ligand shell volume [31]. This shape effect has been exploited for selective end-to-end binding of Au NRs utilizing dithiol linkages[32] or through selective biofunctionalization of the NR tips [33]. More recently, Nie et al. demonstrated self-assembly of amphiphilic Au NRs by carrying out selective tip-modification with a polystyrene-functionalized alkanethiol ligand [34]. Akin to a triblock copolymer, Au NRs were modified to display hydrophobic, polymer-grafted ends and hydrophilic, CTAB-coated side facets. By tailoring solvent mixtures to preferentially solvate the polystyrene-grafted ends, assembly of the NR "triblock" structures could be tailored to form hierarchical architectures such as chains, rings, bundles, and micelle-like structures.

Interaction with biomembranes

Several groups have explored whether the attachment of certain Au-binding ligands enables efficient metal nanoparticle transfection into cells or tissue, with specific attention to how exposed chemical groups at the nanoparticle interface dictate interactions with phospholipid membranes [35,36]. Controlled transport across biomembrane structures is of both fundamental and practical importance given that interaction with the basement membrane dictates the mechanism of cellular uptake. It would be advantageous to engineer nanoparticle surface chemistries that initiate cellular uptake through non-endocytotic pathways such that nanomaterials are not sequestered indefinitely within endosomes. For example, Au nanoparticles functionalized with "striped" ligand shells composed of phase-segregated, mixed alkanethiol monolayers have been demonstrated to undergo cellular uptake via direct membrane

penetration [36]. For Au NRs, displacement of CTAB by phospholipid or fatty acid ligands have been sought to create a ligand surface that mimics membrane chemistry while retaining the same passivating effects as CTAB via the formation of a protective lipid bilayer [37]. Upon ligand exchange, the charged phosphate head group exhibits a strong affinity for the Au surface, resulting in full or partial exchange of CTAB molecules. The anisotropic geometry of the NR also presents a unique surface for chemical interaction with biomembranes. Recent theoretical work from Yang et al. found that for anisotropically shaped NRs, the penetration capability across a lipid bilayer is highly dependent on orientation, NR curvature, and contact area with the membrane bilayer [38]. It would be of fundamental interest to investigate how shape-dependent ligand topology can play a practical role in dictating membrane penetration, and possibly in regulating NR entry into cells and cellular compartments (Fig. 3A).

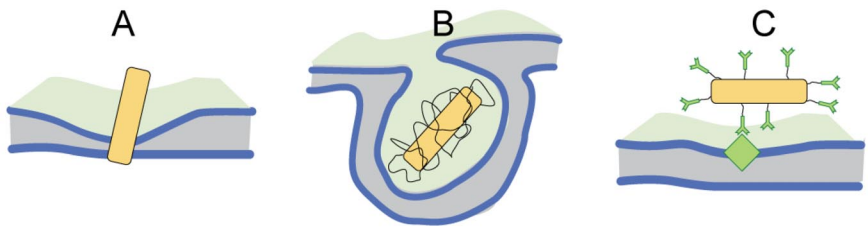

Fig. 3 Schematic of various NR interactions with the cell surface, including: (A) direct penetration; (B) endocytosis; and (C) targeted receptor binding.

LAYER-BY-LAYER DEPOSITION

Layer-by-layer (LbL) approaches have also been demonstrated to effectively coat the NR surface for biocompatibility, without the need for complete displacement of the CTAB bilayer. The LbL approach involves sequential deposition of anionic and cationic polyelectrolytes onto oppositely charged surfaces through electrostatic self-assembly. Because CTAB is positively charged, the first step of the LbL method typically involves adsorption of an anionic polyelectrolyte, such as poly(sodium-4-styrenesulfonate) (PSS) [29]. Several groups have demonstrated that this method is effective at preventing CTAB desorption in vitro, observing decreased cytotoxic effects upon cell incubation with the NRs [39]. In the course of depositing these polyelectrolyte multilayers, small shifts in the SP wavelength are observed due to the change in local refractive index of the NR, whose environment changes from water (n = 1.33) to the polyelectrolyte coating (n = 1.47) [5,40]. Electron microscopy has been utilized to confirm the presence of LbL-deposited polymer coatings, showing uniform polymer layers with a thickness as large as 3 nm [29]. Extending this approach further, the LbL technique can also be used to build composite nanostructures utilizing charged nanoparticles. For example, Gole et al. demonstrated that Au NRs modified with a single layer of PSS could be further coated by citrate-stabilized iron oxide nanoparticles (positively charged) to form a composite NR structure that is mildly responsive to an externally applied magnetic field [41].

The LbL approach can also be used for noncovalent attachment of biomolecules at the NR surface to form a biofunctional or passivating layer. To initiate cellular uptake, Takahashi et al. carried out LbL functionalization of the Au NR surface using alternating layers of bovine serum albumin (BSA) and a cationic polymer [39]. Surface protection with the BSA layers stabilized NR dispersions during incubation with cells and exhibited minimal cytotoxicity during cell uptake. In a similar fashion, Huang et al. utilized the LbL process to generate immunochemical surface coatings for Au NR optical markers, with the end goal of optically tagging malignant cancer cells (Fig. 3B) [1]. For the specific targeting of tumor cells, PSS-coated Au NRs were overcoated with an adsorbed layer of an antibody for epidermal growth factor receptor (EGFR), a cell-surface receptor for proteins that are overexpressed in

malignant cells. In addition to selective binding, these anti-EGFR-coated NRs achieve a higher binding concentration than similarly surface-modified spherical nanoparticles, likely due to the high area of interaction between the NR and the cytoplasmic membrane.

BIOCONJUGATION REACTIONS

Specific biofunctional groups can also be expressed at the NR surface through conjugation chemistry, where Au NRs are functionalized with linker molecules to carry out various conjugation reactions, as depicted in Fig. 4. For example, alkanethiol molecules terminated with carboxylic acid groups provide convenient chemical handles for bioconjugation using carbodiimide coupling to a primary amine (Fig. 4A). This chemistry can be utilized as a powerful tool for biofunctionalization with specific antibodies to develop optical immunoassays for bacteria [4], cell-specific targeting (Fig. 3C) [18], and multiplexed systems [7]. "Click" chemistry has also been demonstrated as a general method for covalently binding biomolecules to NR ligands, where biomolecules must first be covalently modified with the appropriate chemical moiety [42]. For alkanethiol-coated NRs, ligand shell modification with a terminal azide group can then be reacted with acetylene-functionalized biomolecules (Fig. 4B). However, it is unclear how this chemical tethering to the Au NR surface affects the accessibility or activity of certain proteins. Gole et al reported that while carbodiimide methods and "click" methods work equally well to carry out trypsin binding to the NR interface, enzyme activity is significantly lower for NR-trypsin conjugates created via the carbodiimide reaction [42]. As an alternative, Au-binding chemistry can also be carried out using a thiolalkyl-triazole linker to bind peptides and biopolymers directly to the Au surface by insertion into the ligand shell (Fig. 4C) [43].

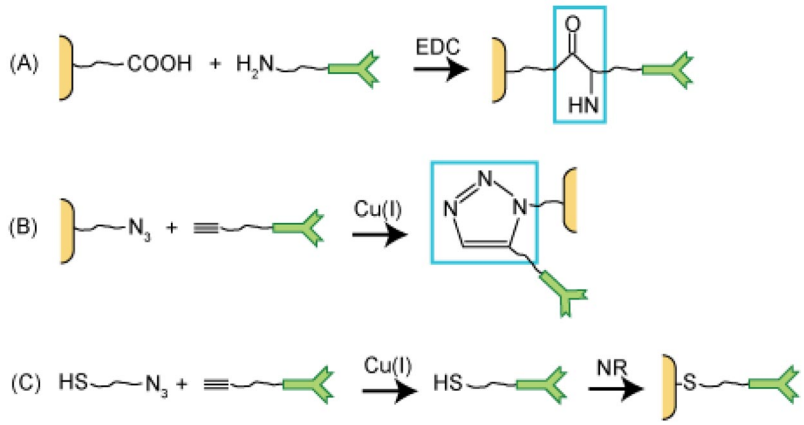

Fig. 4 Schematic of various conjugation strategies for binding specific antibodies to the NR surface, including: (A) carbodiimide coupling with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC); (B) a copper-catalyzed "click" conjugation reaction; and (C) modification of an antibody with a thiolalkyl-triazole linker, prior to incubation with the NR surface.

NONSPECIFIC BINDING

Nonspecific binding at the NR interface has the potential to mask the engineered NR surface chemistries discussed above, presenting a major challenge that must be addressed for the realization of nanomaterial-based biomedical technologies. Largely, nonspecific binding of proteins and other biological macromolecules is difficult to predict and to characterize. For NR-based optical immunoassays, nonspecific binding can compromise detection sensitivity and specificity [5]. These interactions are typically characterized by shorter binding lifetimes and weak interactions with the NR interface, as demon-

strated by protein desorption studies: real-time monitoring of SP wavelength shifts can readily distinguish between specific and nonspecific binding to Au NRs by the observation of drastically different desorption kinetics [22]. However, the SP shifts associated with nonspecific binding typically dictate the lower limit of molecular detection for using these optical readouts.

For applications involving complex biological fluids (e.g., whole blood, serum) or cellular uptake, Au NRs can experience a number of dynamic, nonspecific chemical interactions at the solvent-accessible interface. Such biological environments tend to be crowded, and the intracellular concentration of large biomolecules inside cells is estimated to be in the range of 80–400 mg/ml [44]. For Au nanoparticles, the volume of nonspecifically adsorbed biomolecules—termed the "protein corona"—is largely dependent on the hydrophobicity of the nanoparticle interface and its radius of curvature [45]. Competitive binding between multiple proteins leads to the formation of transient protein–nanoparticle complexes with association/dissociation rates that are highly dependent on preparative conditions. Because nonspecific binding of protein has been linked to denaturation and steric hinderance of active sites, it is possible that these interactions also contribute to nanomaterial cytotoxicity.

"Stealth" coverage

To discourage nonspecific binding, Au NRs are typically functionalized with terminal poly(ethylene) glycol (PEG) ligands. For in vivo applications, these polymer-functionalized "stealth" materials have been shown to suppress immunogenic responses and improve blood circulation lifetimes [9]. For on-chip immunoassays, PEG moieties are thought to not only reduce nonspecific binding, but also behave as spacer molecules to reduce steric hinderance between approaching analyte molecules [5]. While these empirical observations show promise, it is clear that a more fundamental understanding of these interfacial interactions is necessary. The utility of PEG-modified Au surfaces is questionable when faced with the crowded intracellular environment, and it would be beneficial to characterize the degree to which nonspecific binding interferes with NR interfaces engineered for molecular targeting or delivery. Identifying the relevant macromolecules that contribute to the NR corona in a given biological environment may lead to more appropriately designed NR surface chemistries that are programmed to navigate specific biological compartments or tissues. Further, a better understanding of noncovalent association at the Au NR surface may also enable advantageous utilization of nonspecific interactions for designing "smart" materials with dynamic or tunable surface properties.

FUTURE OUTLOOK

The Au NR surface presents an exciting opportunity to create new bio/inorganic interfaces that exhibit highly complex, organized, and functional surface chemistries. As discussed in this review, a number of different chemical methods have been developed to chemically functionalize the surface of Au NRs for biocompatibility; these range from passive surface coatings to biofunctional surface ligands that actively target proteins. Ligand exchange with Au-binding molecules and the LbL method are established protocols that address the presence of CTAB bound to the NR surface postsynthesis, where CTAB desorption can result in cytotoxic and aggregation effects upon NR incubation with tissue or cells. However, there is significant room for progress in advancing the chemical toolbox for NR surface modification, including further study of shape-dependent biofunctionalization and the engineering of nonspecific binding interactions. A greater knowledge of how these Au NR interfaces can be engineered to interact with various biological environments should lead to the increased utility of inorganic nanomaterials in biotechnology and biomedical applications.

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