Designing Plasmonic Nanomaterials as Sensors of Biochemical Transport

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Gold nanoparticle arrays and gold nanorods produce tunable plasmon responses in the near-infrared (NIR) range and can be used to explore biochemical processes in living systems. Colloidal gold nanoparticles up to 170 nm have been self-assembled into periodic 2D arrays when encapsulated by a novel surfactant (resorcinarene tetrathiol). The arrays are capable of supporting cell adhesion, and can be excellent substrates for surface-enhanced Raman scattering (SERS) at NIR excitations. Spherical “superparticle” ensembles (gold nanoparticles densely packed around submicron silica cores) have also been constructed by self-assembly, and exhibit strong NIR extinction and scattering. These core-shell assemblies have been implanted into live cells as intracellular nanoprobes for detecting chemical influx. Third, gold nanorods can be engineered to have longitudinal plasmon resonances at NIR frequencies, and possess outstanding characteristics as optical contrast agents. Gold nanorods can produce strong two-photon luminescence (TPL), and have been imaged in vivo at the single-particle level while passing through a mouse blood vessel. [DOI: 10.1380/ejssnt.2006.9]

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I. INTRODUCTION

Gold nanostructures offer excellent prospects for probing biological systems. The base material is biochemically inert, and can be manipulated at nanometer length scales to support localized surface plasmon resonances at visible and near-infrared (NIR) wavelengths. The latter is well known to penetrate biological tissues with relatively high transmittivities: wavelengths shorter than 800 nm are absorbed by hemoglobin and melanin, and wavelengths greater than 1300 nm are strongly attenuated by water. Plasmonic nanomaterials with NIR resonances are currently being investigated as biomolecular sensors [1, 2], as contrast agents for biomedical imaging [3], and as mediators of photodynamic therapy [4, 5].

NIR-active plasmonic nanomaterials may be especially useful for monitoring biochemical transport events across cell membranes, a vitally important process in physiological function and maintenance. Examples of transmembrane transport include the intracellular delivery of cofactors such as iron and folate, the release and ligand-gated reuptake of neuroactive compounds, and the active export of waste products and cytotoxic compounds. Active transport processes are mediated either by vesicular release (endo- and exocytosis) or by transmembrane proteins. Several transport processes are also critical in the pathology of various disease states; for example, drug efflux pumps such as P-glycoprotein are of particular biomedical importance because of their central role in the “immunization” of drug-resistant tumor cells and pathogens [6]. Methods for monitoring chemical transport across cell membranes are essential for advancing our mechanistic understanding, and for providing rapid and accurate pharmacokinetic analyses of potential drug candidates.

Although significant progress has been made in identifying key pathways pertaining to biomolecular transport, much is still unknown about the temporal relationship between signal and release, or the intracellular transport of compounds after uptake. Real-time assessment of chemical transport is essential for determining the pharmacokinetics of neuroactive compounds or the efficiency of detoxification by multidrug-resistant tumor cells. At present, many transport studies are conducted offline using reagent-based assays and take on the order of minutes. In vitro or in vivo analyses with temporal resolutions on the order of seconds or less could provide a more precise correlation between cell signaling and chemical transport events.

In this paper we will describe several recent advances in plasmonic nanomaterials engineering, and progress in their application as sensors of biochemical transport based on surface-enhanced Raman scattering (SERS) and two-photon photoluminescence (TPL). Colloidal gold nanoparticles in the 20-200 nm size range have been organized into planar arrays and spherical core-shell ensembles using self-assembly techniques. These nanostructures exhibit optical properties which vary as a function of unit particle size and interparticle coupling, and are highly SERS-active at NIR frequencies. Gold nanorods also have strong NIR responses as a function of their aspect ratio, and are capable of plasmon-enhanced TPL. Single nanorod emissions can be readily detected, making them outstanding contrast agents for real-time in vivo imaging.

A. Surface-enhanced Raman scattering (SERS)

SERS is generated primarily by strong electromagnetic fields near the surface of plasmonic nanostructures, which can amplify the Raman scattering cross sections of incident molecules by many orders of magnitude. Single-molecule SERS detection is possible under certain circumstances, and has been demonstrated both with organic molecules and proteins such as hemoglobin [7–9]. Spec-

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troscopic sensing mechanisms such as SERS provide an alternative paradigm to biosensors based on the modulation of linear electronic or fluorescence signals [10–12]. The information content of SERS is high, as the analytes provide a unique vibrational signature intrinsic to their molecular structures, and do not require special labeling or receptor schemes. SERS can also be performed in water, and its response time is limited essentially by the strength of the analyte signal. For example, physiological concentrations of dopamine have been detected at times as short as 25 ms [13].

The integration of high chemical sensitivity with spectroscopic information content by SERS clearly has tremendous potential for biosensing, and numerous applications have been reported in the literature [10, 11]. However, the development of substrates with stable and reproducibly high SERS activity remains a serious challenge. Conventional SERS substrates have disordered nanoscale features, such as those produced by the electrochemical roughening of gold or silver, or the kinetic precipitation of colloidal metal nanoparticles [14, 15]. More recently, nanopatterned surfaces have been fabricated and investigated as SERS substrates, including silver-coated nanosphere arrays [16, 17], submicron gratings [18, 19], pyramidal silver islands [20], and metal films with nanopatterned cavities [21]. Rational substrate engineering holds much promise to develop SERS into a reliable method for ultrasensitive detection and analysis.

B. Two-photon luminescence (TPL)

Nonlinear optical processes such as TPL are attractive for real-time imaging of biological samples with three-dimensional spatial resolution [22]. Modalities based on linear fluorescence or optical scattering are essentially limited to 2D imaging, but TPL signals are also resolved in the axial direction because of their nonlinear dependence on excitation intensity. TPL is typically investigated using fluorescent molecules or nanomaterials with high quantum efficiencies [23], but metals such as gold are also capable of photoluminescence. This has been described as a three-step process: (i) excitation of electrons from the d- to the sp-band to generate electron-hole pairs, (ii) scattering of electrons and holes on the picosecond timescale with partial energy transfer to the phonon lattice, and (iii) electron-hole recombination resulting in photon emission [24]. Luminescence from gold has the disadvantage of low photoemission efficiency, so at first glance gold nanoparticles would appear to be an unlikely choice for strong TPL. However, TPL signals from roughened metal substrates can be amplified by several orders of magnitude by their resonant coupling with localized surface plasmons [25]. This suggests that plasmon-resonant nanostructures can be optimized to produce strong TPL signals using NIR excitation, and thus find use as contrast agents in nonlinear optical imaging. Coupling TPL-active nanoparticles with a robust method of ligand functionalization will further enable their application toward the study of a variety of intracellular processes, as well as site-directed in vivo imaging.

II. GOLD NANOPARTICLE ARRAYS

We have developed self-assembly methods which substantially extend the applicability of nanometer-sized metal particles as engineering materials [26]. Nanoparticles have size-dependent physical properties and play a central role in nanoscale science and technology, but their dispersions are often metastable and require surfactants for stabilization and processing control. Chemisorptive surfactants such as alkanethiols have been successful in stabilizing and dispersing small (< 10 nm) metal nanoparticles in nonaqueous media, enabling the fabrication of two-dimensional (2D) nanoparticle arrays with tunable optical or electronic properties [27]. Larger metal nanoparticles also have size-dependent physical properties, but alkanethiol-encapsulated particles greater than 10 nm do not form thermodynamically stable dispersions.

| resorcinarene tetrathiol 1 | gold nanoparticle |
|-----------------------------|-------------------|
| (diameter=1 nm)             | (diameter=16-170 nm) |

FIG. 1: Resorcinarene-encapsulated gold nanoparticle.
FIG. 2: Self-organized 2D arrays of colloidal gold nanoparticles [35]. (a) 16 nm; (b) 34 nm; (c) 87 nm; (d) average particle diameter \(d\) versus interparticle spacing parameter \(\delta\), based on TEM image analysis. Errors equal one standard deviation.

and precipitate at ambient temperatures [28, 29]. This behavior is due in part to the rapid increase in the van der Waals forces between particles as a function of their size [30], but entropic effects also play an important role. In the case of alkanethiol-stabilized particles, the hydrocarbon chains spontaneously assemble into densely packed monolayers on the nanoparticle facets, which reduce the configurational entropy of the surfactant layer and minimize the subsequent steric repulsion between nanoparticles [31]. Such processing handicaps have substantially hindered the use of colloidal metal nanoparticles in materials applications.

In order to increase the dispersion control of nanoparticles in the mid-nanometer (20-200 nm) size range, we have developed a novel macrocyclic surfactant derived from a class of compounds known as the resorcinarenes (see Fig. 1) [32]. These compounds are well suited for stabilizing nanoparticles: first, the relatively large resorcinarene headgroup (~1 nm in diameter) reduces the surfactant/particle ratio and consequently the entropic cost of encapsulation; second, the macrocycle can be derivatized with multiple chemisorptive functional groups for multidentate binding to the particle surface; third, the macrocycle is appended by several hydrocarbon tails spaced apart in such a manner that their conformational entropy is high even under close-packed conditions. This increases the entropic steric repulsion between nanoparticles and enhances their dispersibility under a variety of conditions. For example, we have demonstrated that gold particles up to 87 nm can be dispersed in nonpolar solvents when stabilized by resorcinarenes [28, 29, 33, 34].

Both the headgroups and tailgroups of the resorcinarene surfactants can be engineered by organic synthesis to have different chemisorptive and dispersive properties. The chemisorptive headgroup determines the degree of surface passivation and influences the electronic polarizability of the metal particle, whereas the tailgroups enhance short-range steric repulsion and modulate the effect of other surface forces. These separate features have enabled us to design amphipathic surfactant layers for promoting the spontaneous organization of nanoparticles into monoparticulate films at the air-water interface. We have recently demonstrated this by using resorcinarene tetrathiol 1 to encapsulate gold particles as large as 170 nm in diameter, which can then self-assemble at the air-water interface and be transferred onto hydrophilic surfaces as highly ordered 2D arrays (see Fig. 2) [35]. Careful analysis of the transmission electron microscopy (TEM) images reveals an inverse correlation between array periodicity and interparticle spacing \(\delta\), most likely as a result of the increase in van der Waals attraction with unit particle size.

The large gold nanoparticle arrays exhibit a number of size-dependent optical properties. Optical extinction spectra indicate that their plasmon resonances shift by several hundred nanometers toward NIR wavelengths with increasing periodicity (see Fig. 3, left). The size-dependent shift in the plasmon bands of the 2D arrays are much more pronounced than that of individual metal nanoparticles, because the electromagnetic couplings be-
FIG. 3: Left, Extinction spectra of gold nanoparticle arrays on quartz substrates [35]. Right, gold nanoparticle arrays viewed at (a) normal and (b) 60° angles of incidence [41]. Substrate width is approximately 1 cm.

FIG. 4: Tunable SERS activity from the gold nanoparticle arrays [41]. (a) Raman spectra obtained using a dispersive spectrometer operating at 647 nm; (b) Raman spectra obtained using a FT spectrometer operating at 1064 nm; (c) signal enhancement factors (G) as a function of periodic structure and excitation wavelength; (d) G values as a function of numerical aperture at a fixed excitation wavelength (785 nm).

tween particles are highly sensitive to structural changes [36–40]. The arrays also vary in their optical reflectivities, with the maximum specular reflectances being produced by arrays of intermediate periodicity (see Fig. 3, right) [41]. The smaller nanoparticle arrays are significantly absorptive at optical frequencies, whereas the reflectivities of the larger nanoparticle arrays are diffused by Mie scattering.

The gold nanoparticle arrays demonstrate highly stable and reproducible SERS with surface-averaged signal enhancements ranging from $10^4$ to over $10^7$ (see Fig. 4) [41]. These activities are tunable as a function of periodicity as well as excitation wavelength. Empirical enhancement factors (G) were determined by measuring the ratio of Raman spectral intensities from adsorbed tetrathiol 1 to the corresponding unenhanced spectral intensities from thin films at 647, 785, and 1064 nm excitation. Overall, the SERS activities correlate strongly with both unit particle size and excitation wavelength, similar to the size-dependent shifts in plasmon resonance. These observations are in accord with theoretical calculations describing electromagnetic SERS [42–46] and also with earlier SERS studies on disordered metal colloid aggregates [47, 48]. Additional signal enhancement could be obtained by increasing the solid angle of incidence and collection; changing the numerical aperture of the objective from 0.25 to
0.75 increases $G$ by nearly an order of magnitude, after adjusting for changes in sampling power (see Fig. 4d). The aperture-dependent Raman intensities suggest that surface plasmon polaritons, whose angular dependency on the electric field is well known [49, 50], contribute significantly to the SERS effect in the gold nanoparticle arrays. The nanoparticle 2D arrays could retain essentially all SERS activity more than a year after self-assembly, and demonstrated considerable advantages in reproducibility and stability over random nanoparticle aggregates.

The Au nanoparticle 2D arrays are also capable of detecting exogenous analytes by SERS, although their signals were not as strong as those from the surfactants themselves. Theoretical studies on closely packed nanoparticles have indicated that the electromagnetic field enhancements responsible for SERS are highly localized between particle surfaces (see Figure 5) [51, 52]. These local SERS intensities can be many orders of magnitude greater than the surface-averaged enhancements, making self-assembly an appealing method to develop well-defined 'hot spots' for ultrasensitive SERS spectroscopy [53]. On the other hand, restricted access to these highly active sites limits the usefulness of the close-packed 2D arrays for analyte detection.

Recent theoretical calculations on periodic 2D arrays of metal nanoparticles have suggested strategies for further improving analyte sensitivity by adjusting the particle diameter-spacing ratio (see Fig. 5) [52]. Resonant $G_{av}$ values from 2D arrays can be several orders of magnitude greater than those produced by disordered metal aggregates. In addition to field optimization, adjusting the spacing $\delta$ also increases the available sampling volume for analyte detection, a current limitation of the close-packed arrays. For example, the $G_{av}$ values from 100-nm particle arrays can be optimized for spacings between 3-10 nm, providing sufficient space for the infiltration by exogenous chemical species.

The nanoparticle arrays are excellent substrates for cell adhesion (see Fig. 6). We have tested several cell lines (HeLa, HEK 293, and β-pancreatic cells) for compatibility with the nanostructured substrates and for robustness of adhesion. In all cases, the cells bonded strongly to the substrates and remained viable for days at a time; detachment of the cells could only be achieved by enzymatic cleavage using trypsin. The arrays do not seem to be adversely affected by cell adhesion: negative con-
trol experiments indicate that the arrays retain the same level of SERS activity after cell attachment, and the cells themselves do not produce any appreciable Raman signals when attached to the substrates. This strongly suggests that the SERS activity is indeed highly localized in the interstitial regions between particles. Therefore, only those analytes which are small enough to diffuse to those regions of high activity should be detectable by SERS. An intriguing feature of this system is that any analytes which are released toward the arrays will be sealed in by the cell membrane. This greatly increases their effective concentration and the subsequent limits of detection: a single molecule trapped in a subattoliter cavity defined by three spherical 100-nm particles covered by the cell membrane ($\sim 10^{-19}$ L) will have an effective concentration on the order of 20 $\mu$M, a concentration well within our means to detect.

III. SPHERICAL GOLD NANOPARTICLE ENSEMBLES

As a complement to the gold nanoparticle arrays above, we have developed electrostatic self-assembly methods for fabricating spherical core-shell assemblies of densely packed gold nanoparticles on submicron silica particles, as intracellular nanoprobes for detecting chemical influx events using SERS. These “superparticles” bear some resemblance to the semicontinuous gold nanoshells reported by Halas [54–57], but differ with respect to their plasmon modes. In the case of the superparticles, field enhancements would be generated primarily by electromagnetic coupling between discrete nanoparticles. Again, we wished to utilize the strong plasmon resonances of colloidal gold nanoparticles in the 20-80 nm size range, whose polarizabilities and strong van der Waals interactions should promote high shell densities.

Dilute aqueous suspensions of submicron silica particles (330-650 nm) functionalized with high molecular-weight polyethyleneimine were combined with citrate-stabilized gold nanoparticles (20-80 nm) in nearly stoichiometric ratios [58]. The shell density of gold particles on the silica core was determined by TEM and approximated as $n\Omega/2\pi$, where $n$ is the observable number of gold particles in a hemispherical ensemble and $\Omega$ is solid angle occupied per nanoparticle. Surface potential was found to be a critical factor for high shell coverage, which was optimized to densities as high as 55% (see Fig. 7). These packing densities are a significant advance over previous reports of core-shell nanoparticle assemblies, nearly all of which have been formed with low or non-uniform particle coverage.

The gold-silica superparticles exhibit bimodal extinction maxima, generated presumably by individual and
collective plasmon responses, with shell layers of 40 nm or
more producing significant extinction in the NIR region
(see Fig. 8, left). Both elastic scattering and SERS intensi-
ties are high for NIR excitation wavelengths, and appear
to be at least two orders of magnitude greater than that
produced by monodispersed nanoparticles. SERS spectra
of the superparticles were taken at excitation wavelengths
of 785 nm; the peaks recorded are due to adsorbed citrate
(see Fig. 8, right).

Several different methods for introducing superparticles
into live cells have been investigated. Nanoparticles were
first introduced into cells by picoliter injection using cus-
tomized micropipettes, with co-injection of GFP-encoded
plasmids to assess cell viability. Individual 100-nm gold
particles were used to test the feasibility of this implan-
tation method, using an epipolarization filter to detect
particle-induced light scattering. Cell survival rates were
approximately 25% based on GFP expression 24 hrs after
injection; cell swelling was initially observed as a trauma
response, but subsided after 1-2 days. However, the mi-
cropipets became easily clogged with nanoparticle aggre-
gates, which precluded the routine use of this method for
nanoprobe implantation.

Nanoprobes are more reliably introduced into cells us-
ing a cationic transfection system, which causes minimal
cell trauma (see Fig. 9a) [59]. Co-transfection of super-
particle nanoprobes and GFP plasmids into tsA201 cells
followed by 1-3 days of incubation resulted in a healthy
increase in cell population, with an implantation success
rate of approximately 10%. The nanoprobes can be de-
tected in more than 90% of the GFP-expressing cells 24
hours after transfection. Very importantly, the implanted
superparticles do not appear to impair normal cell growth
or function. The nanoprobes can even be passed on by mi-
tosis to daughter cells for several generations, validating
their retention within cells as well as their biocompatibil-
ity. Lastly, NIR excitation of the intracellular superparti-
cles produced strong SERS signals from adsorbed DNA,
with a comparable intensity produced by single superpar-
ticles on glass slides (see Fig. 9b) [60]. Further explo-
FIG. 10: Sulfide-arrested growth of gold nanorods [65]. (a) Gold nanorods without sulfide treatment experience an "optical drift" in LPR of up to 100 nm toward shorter wavelengths. (b) Treatment with Na₂S effectively arrests the LPR blueshift, and stabilizes the nanorods at NIR wavelengths.

FIG. 11: (a) Still image of TPL signals from single gold nanorods (marked with red arrows), passing through the blood vessel in a mouse ear [66]. The blood vessel walls have been highlighted for clarity.

IV. GOLD NANORODS AS TPL CONTRAST AGENTS

Gold nanorods are appealing as NIR optical contrast agents because their longitudinal plasmon resonances (LPR) can be tuned as a function of their aspect ratio. Nanorods can support a more sustained plasmon resonance than spherical nanoparticles due to reduced damping effects, which scale as a function of particle volume [61]. It has been shown that gold nanorods are capable of photoluminescence, and that their quantum efficiencies increase enormously under plasmon-resonant conditions [62]. These qualities suggest that gold nanorods should also be good candidates for producing enhanced TPL signals, with subsequent application toward nonlinear optical imaging.

Gold nanorods were prepared by the reduction of AuCl₄⁻ in micellar solutions of cetyltrimethylammonium bromide (CTAB, 0.2 M), seeded by smaller spherical gold nanoparticles with AgNO₃ as an additive [63, 64]. However, we observed that the LPRs of these nanorods were unstable, drifting toward shorter wavelengths by as much as 100 nanometers over a period of hours to days. This "optical drift" is due to the slow reaction of unreduced AuCl₄⁻ still strongly associated with the CTAB micelles, and is effectively arrested by treating the nanorod suspensions with millimolar concentrations of Na₂S [65]. The sulfide-treated nanorods are indefinitely stable at room temperature after isolation from the reaction mixture, shifting less than 5 nm over time (see Fig. 10). Sulfide treatment provides an additional benefit by shifting the LPRs toward longer wavelengths, which can be ascribed to modulations in the dielectric function (i.e. increase in the refractive index) at the nanorod surface. In contrast to the slow optical drift described above, the sulfide-induced redshift is essentially a singular event, resulting in stable NIR resonances for nonlinear optical imaging applications.

Gold nanorods were deposited onto glass cover slips and excited at their LPR wavelength (\(\lambda_{\text{max}} = 830\) nm) by a far-field laser-scanning microscope. Strong TPL intensities were observed from single nanorods with a \(\cos^4\) dependence on the incident polarization, indicating an orientational dependency on a single dipole [66]. The TPL excitation spectrum could be superimposed onto the LPR band, indicating a plasmon-enhanced two-photon absorption cross section. In contrast, the emission band was broad and essentially depolarized, confirming the incoherent nature of TPL. Comparing the nanorods’ TPL intensities with that from single rhodamine dye molecules reveals a nearly 60-fold difference in brightness; the two-photon action cross section of the average nanorod is estimated to be more than 2000 GM.

The nanorods’ plasmon resonance in the NIR region makes them ideal probes for TPL imaging in biological tissue [66]. Picomolar concentrations of nanorods were
delivered into an anesthetized mouse by tail vein injection, followed by monitoring of the earlobe vessels. Intermittent TPL signals could be detected within a few minutes, with a brightness approximately 3 times greater than the background autofluorescence from the blood and surrounding tissue (see Fig. 11). The uniform intensities of the TPL signals in single-frame images indicate that the great majority of these were produced by single nanorods. It is worth noting that the gold nanorods are essentially inert to destructive photobleaching effects, which has important practical consequences in biophotonic applications.

V. CONCLUSIONS

The examples above represent some of the many opportunities for designing plasmonic nanomaterials for applications in analytical biochemistry and biological imaging. An important next step is to functionalize these substrates using a robust method of surface attachment, one which is compatible with biological systems. Recent studies from our group have shown that a variety of alkyllamines can be anchored onto gold surfaces as dithiocarbamates (DTC) by simple condensation with CS₂ [67]. DTC ligands are able to resist displacement by organic thiols, which strongly suggests their robustness under physiological conditions in the presence of biogenic thiols such as cysteine and glutathione. We have recently functionalized gold nanorods with a folate derivative using DTC ligation, and have observed its rapid uptake into tumor cells by receptor-mediated endocytosis [68]. This synergy between plasmonic nanomaterials engineering and novel surface functionalization methods is anticipated to lead to further advances in our efforts to investigate questions of biochemical transport.

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