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Super Robust Nanoparticles for Biology and Biomedicine

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Nanoscience has had an impact on almost every branch of natural science, in particular materials science, chemistry, physics, biology, and medicine. Some of the significant and most promising applications of inorganic nanoparticles lie in the fields of biology and biomedicine,[1–3] for example, by using nanoparticles as labels for DNA and protein detection, as probes for magnetic resonance imaging and dynamics studies in biological systems, as well as tracers for the localization of marker proteins. A major issue with the development of inorganic nanoparticles for biological applications pertains to the stability of bio-functionalized nanoparticles in biological media.[1,2] This has a direct impact on the sensitivity, selectivity, and nonspecific properties of all nanoparticle-based biosensors. Previous work on gold nanoparticles functionalized with thiolated DNA constitutes a milestone in the development of robust nanoparticles for biology.[1] Recently, Cao and co-workers reported a further improvement in particle stability, which constitutes a new promising advance.[4]

Historically, the utilization of nanoparticles (for example, gold colloids) for aesthetic and curing purposes dates back more than two thousand years ago to ancient China and Egypt.[5] The "soluble" colloidal gold appeared around the 5th century B.C.[5] In the early 1950s, the successful determination of the double-helical structure of DNA triggered a revolution in biology that led to the birth of modern molecular biology. A major motivation for the application of nanoparticles in biology is that transmission electron microscopy (TEM) started to become popular after World War II: Molecular biologists could then use TEM to observe high-resolution cellular structures and constituents down to the nanometer scale. A major problem encountered, however, was the low contrast of unlabeled biological tissues under TEM, which is due to the lower electron scattering capabilities of the light elements C, H, N, and O. (The electron-scattering capability of atoms through electron–nucleus interactions scales as the square of the atomic number Z according to $\sigma \propto Z^2$, where σ is the scattering cross-section.)

To overcome the problem of contrast, a staining technique was developed in which compounds of heavy metals such as tungsten, mercury, uranium, lead, and osmium were used. In 1945, Porter et al. used TEM to examine cells in tissue cultures after staining them with OsO_4.[6] The various staining techniques are still being widely used nowadays. However, a major drawback of using biomolecules derivatized with heavy-metal compounds is that such complexes are still not capable of providing sufficiently large electron-scattering signals to be distinguished as individual biomolecules by conventional TEM techniques or advanced scanning TEM techniques (STEM Z-contrast imaging, resolution ca. 0.1 nm after correction for spherical aberration). Thus, visualization of ultrastructures down to the single-molecule scale in biological systems has been problematic until now.

During the 1970s researchers started to look at the field of colloid science. In 1971, Faulk and Taylor were perhaps the first to use colloidal gold as an electron-dense immunoprobe.[7] Subsequently, Romano et al. developed gold-labeled antibodies in 1974.[8] It is noteworthy that in much earlier studies dating back to 1959, Singer used ferritin (a protein containing a ferric hydroxide-phosphate core with a diameter of ca. 10 nm) to label antibodies.[9] Nonetheless, it was not until the 1970s that extensive research started to be carried out to develop suitable chemical techniques to make electron-dense, water-soluble nanoparticle labels.[10–12] Such nanoparticles provide better resolving power in the TEM studies of cellular ultrastructures, for example, the detection of single protein subunits in a viral capsoid. In the 1980s, gold–antibody conjugates were widely used in histochemistry, immunocytochemistry, and immunopathology.[12,13] New techniques for immunocytochemistry were developed that utilized different sized gold nanoparticles to multiply label samples.[14] With the introduction of small gold nanoparticles (for example, nanogold with a diameter of 1.4 nm, and thus much smaller than IgG molecules with a size of 5–10 nm), a considerable improvement in the permeability of gold markers to antigenic binding sites was achieved.[15]

In most previous cases, the conjugation of gold nanoparticles to biomolecules such as antibodies, lectins, and other proteins relied on nonspecific (for example, adsorption) or noncovalent (for example, electrostatic) interactions between nanoparticle surfaces and biomolecues.[7,8] Thus, the stability

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of such nanoparticle probes were low. This issue is even more crucial in biological studies under harsh experimental conditions, for example, the tracking of biomolecules and dynamics studies over a long time, at high salt concentration, as well as in the presence of attacking molecules such as dithiothreitol (a small, uncharged molecule with two thiol groups, used to protect proteins from oxidation).

In 1996, the research groups of Mirkin and Alivisatos pioneered the preparation of gold nanoparticles functionalized with thiol-terminated DNA oligonucleotides (Figure 1 A).[16,17] The as-prepared conjugates (or probes) allow

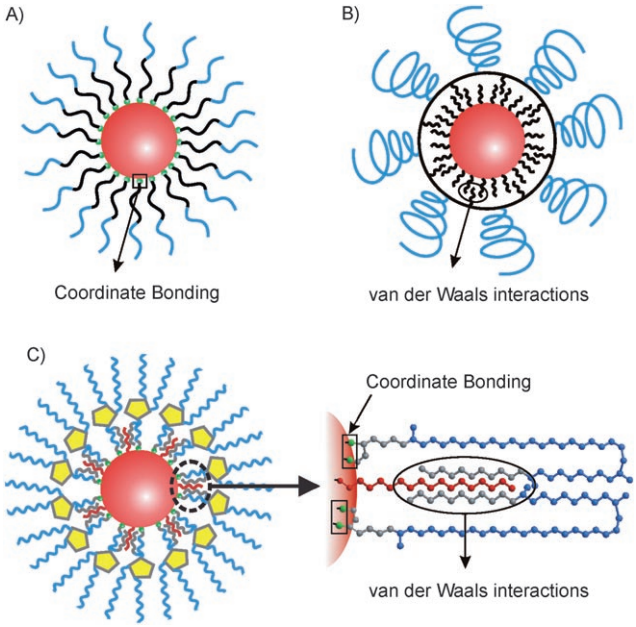


Figure 1. Strategies for stabilizing nanoparticles for biological applications. A) Thiol-terminated ligands (for example, DNA). B) Hydrophobic interactions. C) A versatile strategy for preparing super-stable, water-soluble inorganic nanoparticles.

for the ultrasensitive detection of complementary DNA targets, with the gold nanoparticles serving as the signal reporter.[16] Since then, a number of detection formats based upon such covalently bio-functionalized gold nanoparticles have been developed for protein detection, disease diagnosis, and gene expression, etc.[1] These heavily functionalized nanoparticle probes were demonstrated to be quite robust:[18] they can withstand a very high salt concentration (for example, 2 M NaCl), which is in contrast to colloidal gold (for example, citrate stabilized gold colloids) which is unstable even in the presence of a low salt concentration (ca. 10 mM NaCl); they are extremely stable under thermal conditions (for example, boiling); and they can resist, to some extent, attack by molecules such as dithiothreitol or molecules bearing SH, phosphine, and NH_2 groups.[18a]

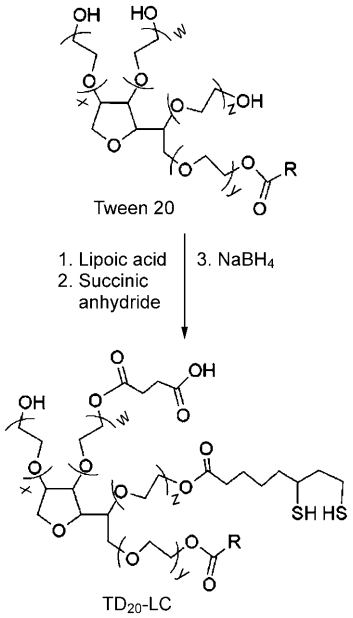
The successful preparation of DNA-functionalized gold nanoparticles constitutes a milestone in the development of robust nanoparticles for biological applications. There are two main mechanisms involved in stabilizing gold nanoparticles: a) thiols possess a high affinity to gold, thereby resulting in a dense coating layer of thiolated DNA on the particle surface; b) the DNA backbone carries multiple negative charges because of the phosphate groups, thereby resulting in a strong negatively charged layer around the nanoparticle that prevents other nanoparticles and charged molecules from approaching. Thus, particle aggregation is prevented, even under high salt concentrations or high temperatures.[16,18] Despite the extraordinary robustness of Au nanoparticles functionalized with DNA, they do have several limitations. For example, these nanoparticles are still somewhat vulnerable to dithiothreitol;[18a] in addition, the thiol method that works well for metal nanoparticles is not suitable for quantum dots and metal oxide nanoparticles such as Fe_3O_4.[3,4]

Quantum dots are promising as a new type of fluorescent label for biomolecules.[2] Since the major breakthrough in synthetic methods for preparing high-quality quantum dots,[19] this type of nanomaterial has attracted significant research interest. Quantum dots offer several advantages over traditional dyes, including size-tunable photoluminescence, a wide range of excitation wavelengths, high quantum yields (>50 %), and good chemical stability. These properties render quantum dots superior to existing labeling reagents such as organic fluorophores (for example, Cy fluorophores) and fluorescent proteins (for example, the green fluorescent protein, GFP).[2,3] However, these high-quality quantum dots are typically made in organic solvents, and coaxing them into aqueous media has been nontrivial, which is a crucial step for biological applications. Another complication is that their high quantum yields in organic solvents drops significantly in water because of the quantum leakage of excitons.[19b] Coating quantum dots with amphiphilic ligands by utilizing hydrophobic van der Waals interactions between the hydrophobic tail of the ligand and the primary ligands on the nanocrystal surface led to the formation of nanocrystal micelles (Figure 1 B).[20] However, the drop in photoluminescence was not fully resolved.

Given the limitations concerning the applications of metal nanoparticles (for example, Au) and quantum dots (for example, CdSe/ZnS), it is of crucial importance to develop new techniques for preparing the most robust nanoparticle probes possible so as to expand their applications to biology and biomedicine. In a recent report, Cao and co-workers devised a versatile strategy for engineering the nanoparticle surface coatings (that is, passivating ligands) to enhance the robustness under harsh conditions.[4] They cleverly combined two types of interactions (Figure 1 C): a) coordinate bonding between the stabilizing ligands and particle surface, and b) van der Waals interactions between the hydrophobic tail (moiety R, gray) of the Tween derivatives and the primary ligands (red) on a nanocrystal surface (see circled region in Figure 1 C). This dual-interaction mode has been demonstrated to significantly enhance the robustness of the resulting nanoparticles, which makes them suitable for biological applications under very harsh conditions. This idea, at first sight, may seem to be analogous to a previous approach devised by colloid chemists to effect hydrophobic van der Waals interactions between the hydrophobic tails of amphiphilic ligands and the primary hydrophobic ligands on the nanoparticle.[20] However, in the new approach, the Tween

derivative ligands coordinate to the nanoparticle surface which leads to smaller nanoparticles than the nanoparticle micelles. Thus, these new nanoparticles offer greater processibility and functionalizability, which is advantageous in terms of the ability of the particle probes to penetrate cell membranes, and is thus of crucial importance for in vivo applications of nanoparticles.

To implement their idea, Cao and co-workers used polyethylene glycol (PEG) sorbitan fatty acid esters (commercial name: Tween; for an example, see Scheme 1) as



Scheme 1. Synthesis of a carboxy-functionalized Tween ligand (TD_{20}-LC) from Tween 20 ($w+x+y+z=20$, $R=C_{11}H_{23}$).

scaffolds. This molecule has four arms, one of which has a long-chain hydrophobic moiety (R). Tween can have different numbers of ethylene glycol units (for example, $n=20$ units for Tween 20). By using the Tween scaffolds, Cao et al. were able to synthesize new difunctional ligands. The resulting Tween derivatives (TD_n) show strong affinity to hydrophobic nanoparticles through coordinate bonding as well as hydrophobic van der Waals interactions. This new type of TD_n ligand successfully overcame the problem that the Tween arm itself can only exert weak van der Waals interactions with the hydrophobic ligands on the particle surface. Such ligands (TD_n, $n=20, 40, 60, 80$) have been utilized in preparing water-soluble, super-stable nanoparticles of Au, CdSe/ZnS, and Fe_3O_4. The as-functionalized gold nanoparticles show extraordinary stability in a wide pH range (pH 1–14) and under high salt concentrations (up to 5 M NaCl).[4] Such unprecedented stability is even greater than that of gold nanoparticles functionalized with thiol-DNA, which points to the possible use of such "super robust" nanoparticles in biology and biomedical applications.

A major advantage of the difunctional ligand is that it successfully resolved a long-standing problem, namely, the high fluorescence quantum yield of the quantum dots was retained after transferring into aqueous media. Cao and co-workers demonstrated that CdSe/ZnS nanoparticles, after being transferred into aqueous media, retained high quantum yields (ca. 50%) for more than three months under extreme conditions (pH 2–12.5).[4] It is noteworthy that the extraordinary stability of TD_n-functionalized QDs is significantly higher than that of quantum dots functionalized with PEGylated lipoic acid ligands; these ligands lack hydrophobic interactions with the nanoparticle surface. The PEGylated polymer shell results in a large hydrodynamic diameter of 30–40 nm, which is not desirable, as it limits their use in in vivo cell imaging because such large nanoprobes are less capable of penetrating into cells and tissues. In contrast, the TD_n-functionalized quantum dots show a much smaller hydrodynamic diameter (<20 nm).

Another advantage of the Tween scaffold is that it allows a carboxy group to be readily introduced into one of the available TD_n arms. This permits easy attachment of biomolecules such as antibodies through a mild coupling reaction mediated by *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC).[4] This versatility is not achievable with other types of simple molecule ligands such as long-chain alkanethiols.[20d] The ability to modify more than one arm of the Tween scaffolds on the nanoparticle surface can result in cooperative properties that can lead to enhanced binding of the target molecule and the introduction of a variety of functional groups, and provide information on how the structure works within a cell.

These robust nanoparticles of Cao and co-workers are less susceptible to degradation by nuclease activity, less or non-toxic to the cells, and have greater cellular uptake because of their smaller size. Thus, they hold promise in many applications, such as intracellular gene regulation agents for the control of protein expression in cells, as a vector (for example, for introducing drug molecules) to specific cell types and different components within the cell compartments. The preparation of ultrastable nanostructures with as small a size as possible but with a large number of signal reporting groups and with the versatility to perform surface reactions on nanoparticles will continue to be a central topic in the field of nanobiotechnology. Such robust nanoparticles will afford new possibilities in the study of gene function and nanotherapies.

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