On the Development of Colloidal Nanoparticles towards Multifunctional Structures and their Possible Use for Biological Applications

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Keywords: 
- biological systems
- colloids
- DNA
- hybrid materials
- nanoparticles
In this Review, we describe the synthesis of high-quality colloidal nanoparticles in organic solvents, the mechanisms by which they can be transferred into aqueous solution, and some of their applications in biology. In particular, we will place emphasis on the creation of multifunctional nanoparticles or nanoparticle assemblies.

1. Introduction

Nanocrystals represent one type of artificial nanostructure that can be designed to exhibit different properties. For example, semiconductor nanocrystals composed of materials such as CdSe, CdTe, and InP can be physically described as quantum dots,[1,2] which exhibit atom-like energy states that are a consequence of the confinement of carriers in three dimensions. Due to their particular electronic properties they can be used, for example, as active materials in single-electron transistors.[3] The atom-like energy states also contribute to special optical properties, such as a particle-size-dependent wavelength of fluorescence. Furthermore, nanocrystals composed of magnetic materials such as Co, CoPt,

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and Fe2O3 possess magnetic properties: each particle can be regarded as a single nanomagnet. Depending on their material and their shape, nanocrystals can possess many varied properties. Thus, we can consider nanocrystals as functional building blocks on the nanometer scale.

Such tiny building blocks are particularly interesting with regard to the construction of smaller and faster devices or multifunctional materials on the nanometer scale. However, each building block is itself isolated, thus to form a device or multifunctional entity the building blocks have to be arranged and connected. Since a large number of building blocks are required to form devices with reasonable complexity, the process of arranging them must be carried out in a parallel way, rather than in a sequential fashion. This is where biological molecules come into play. Certain biological molecules have two important properties: they are capable of molecular recognition and they can self-assemble. Molecular recognition is a “key/lock” principle realized on a molecular scale: Receptor molecules (the “lock”) recognize certain ligand molecules (the “key”) with very high selectivity. Thus, only the appropriate ligand will bind to its receptor. Several important classes of receptor–ligand pairs exist, such as oligonucleotides and their complementary counterpart, antibodies and antigens, and the biotin/avidin system.[4]

The self-assembly process dictates that molecules find their appropriate positions without any external driving force. The idea is to harness these properties of biological molecules to control the arrangement of building blocks on the nanometer scale. For this purpose, each building block has to be functionalized with ligand molecules. The building block–ligand conjugates will now bind to positions where corresponding receptor molecules are present (see Figure 1). In this way the following three types of applications are possible: 1) The assembly of receptor–ligand-mediated groupings of building blocks[5–7] to form new multifunctional building blocks, 2) the arrangement of ligand-modified building blocks on a surface that is patterned with receptor molecules,[8–15] and 3) the labeling of specific receptors in a cell with ligand-modified building blocks.[16–18]

1) The first application will allow for the creation of new materials with multifunctional properties.[19] Let us assume that we have two types of building blocks characterized by different properties. In order to combine both properties, each building block of one type must be functionalized with one ligand molecule, and each building block of the other type must be functionalized with the corresponding receptor molecule. If the two different building blocks are mixed together, they will recognize each other and bind to form a new entity that combines the properties of both starting blocks. It should be pointed out that the processes described here are solution-based and can occur in parallel for a large number of building blocks. Despite its simplicity, the assembly is precisely controlled at the nanometer scale.

2) One example for the second application is the assembly of single-electron transistors on a substrate to designated positions. Although problems concerning the arrangement of the components of a nanocircuit could be solved in this way, the problem of connecting or wiring the individual components remains. However, solutions for this problem based on biological molecules have also been demonstrated. With the same principles of molecular recognition and self-assembly, individual building blocks can be connected with DNA molecules. Upon metallization, the DNA molecules become conductive and can act as molecular wires.[20–23]

3) The labeling of cellular structures with antibodies that specifically recognize a designated structure is a common method in cell biology.[24] Typically, antibodies
are conjugated to an organic fluorophore, so that the labeled structure can be visualized by fluorescence microscopy. Instead of conjugating the antibodies to organic fluorophores, they can be conjugated to any nanoscale building block. Depending on the properties of this building block, improved and different ways of visualizing the labeled structures are possible.[25,26]

In this article, we will describe colloidal inorganic nanocrystals as a versatile example of nanoscale building blocks. In most cases, these building blocks are synthesized most successfully in organic surfactants. As a consequence of this synthetic approach, they are often hydrophobic. There are methods for transferring these nanocrystals into aqueous solution, which will be reviewed herein. As a next step, the nanocrystals have to be functionalized with biological molecules; an example of how small groupings of nanoparticles can be formed using these conjugates will be provided. Finally, a discussion on the uptake of colloidal nanocrystals by living cells is given, as well as an outlook on future possibilities, in particular the creation of multifunctional structures.

2. Nanoparticle Synthesis in Organic Solvents

Our groups use inorganic colloidal nanocrystals as one possible type of nanoscale building block. Colloidal nanocrystals are crystalline clusters of a few hundreds up to a few thousands of atoms, which are dispersed in a solvent. In other words, each colloidal nanocrystal is an individual, freestanding nanoparticle in solution. Inorganic colloidal nanocrystals can be prepared from many different materials, including metals, semiconductors, and insulators (Figure 2).
A large variety of methods exist for growing nanocrystals in aqueous solution, and as such those methods deliver nanocrystals that are hydrophilic and water-soluble. However, growth in a specific organic medium that acts as a stabilizer in the absence of water is often preferred. Such organic media are called surfactants and are composed of molecules that exhibit a polar head group and one or more hydrocarbon chains, which constitute the hydrophobic part of the molecule. One important advantage of growing nanocrystals directly in organic surfactants is that several surfactants can be heated well above 100°C (the boiling point of water). The use of high temperatures and the absence of water expands the range of materials that can be synthesized, and indeed, the optimal combination of surfactants and reaction temperatures suitable for growing a given material can be ascertained, as explained in more detail below. In addition, various defects in the crystal lattice of nanoparticles, which can form during synthesis, can be annealed out easily at higher temperatures.

Surfactants are crucial for the controlled growth and stability of nanocrystals. In the case of nanoparticles directly grown in organic surfactants, each nanocrystal is coated with a monolayer of surfactant molecules, which are bound to the surface of the nanocrystal via their polar head groups, while exposing their hydrophobic tails to the outer environment (see Figure 5). This outer layer of surfactants effectively renders the nanocrystals hydrophobic, and as such they can be easily dissolved in a wide range of nonpolar or moderately polar organic solvents. Van der Waals interactions would favor interparticle aggregation, however, the presence of the stable organic coating prevents the inorganic cores of neighboring nanocrystals from touching each other, thus resulting in a solution of well-dispersed particles. This can be deduced easily by observing a monolayer of surfactant-coated nanocrystals deposited on a suitable substrate, viewed under a transmission electron microscope (TEM, see the left column of Figure 2). This image shows a sample of nanocrystals that were prepared by casting a drop of solution containing the nanocrystals on a thin film of amorphous carbon and by allowing the solvent to evaporate. Here a gap can be seen between adjacent nanoparticles, which is due to the presence of the organic coating layers that act as spacers between adjacent nanocrystal cores. Due to intrinsic limitations of the TEM technique, these layers cannot be seen directly in the image, and the corresponding region appears as transparent.

Some basic principles of nanocrystal growth can be described by referring to a well-studied case, the growth of CdSe nanocrystals. The discussion, however, will sacrifice some details and peculiarities that are particular to other systems. For a typical synthesis of CdSe nanocrystals, a mixture of surfactants is heated at temperatures of around 250–300°C. The surfactants usually chosen to grow CdSe nanocrystals are a mixture of trioctylphosphine oxide (TOPO) and a phosphine, such as tributyl- or trioctylphosphine. This mixture can also be more complex, and include alkyl amines, phosphonic acids, or carboxylic acids. The atomic species that will form the nanoparticles are added to this solution containing the nanocrystals on a thin film of amorphous carbon and by allowing the solvent to evaporate. Here a gap can be seen between adjacent nanoparticles, which is due to the presence of the organic coating layers that act as spacers between adjacent nanocrystal cores. Due to intrinsic limitations of the TEM technique, these layers cannot be seen directly in the image, and the corresponding region appears as transparent.

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factants, it decomposes and Cd atoms are released. In a similar way, Se atoms are introduced through complexation with a phosphine. At high temperature this complex dissociates to release the Se atoms. Recent advances in the synthesis of CdSe nanocrystals have led to less-hazardous precursors for Cd, such as a complex between Cd$^{2+}$ ions and an organic acid,[132,133] This precursor can be formed directly in the re-action flask by heating together cadmium oxide (or other cadmium salt) with the organic acid.

The surfactant molecules play a key role in the synthesis. They bind to the surface of the growing crystals and, in addition, they form complexes with the atomic species in solution, thus controlling the reactivity and the diffusion of the elemental species to the surface of the growing nanocrystals. Without them, the Cd and Se species would bind rapidly to each other, resulting in uncontrolled growth of CdSe crystals. A common feature of the surfactants used in the synthesis is that their polar head groups are functional moieties capable of donating electron pairs. Hence all of these surfactants can be defined as Lewis bases and the overall influence in controlling the growth of nanocrystals is mainly dictated by their ability to form complexes with the free Cd species in solution and to their binding ability to the Cd atoms on the surface of the nanocrystals.

The stability of the complexes formed in solution between the surfactants and the atomic species, their diffusion coefficients, as well as the binding strength of the surfactants to the nanocrystal surface, are all parameters that vary with temperature. Low temperatures lead to high complex stabilities, low diffusion, and higher surface coordinating strength, all factors that limit or even hinder nucleation and crystal growth. On the other hand, too high a temperature can also lead to uncontrolled growth. Thus choosing an appropriate temperature range is key to the control of particle growth: once a desired average crystal size is reached, the growth can be stopped by simply cooling down the reaction flask below the temperature range at which growth is possible.

The growth of nanocrystals depends on a number of parameters, such as the surface energy of the nanocrystal, the concentration of free species in solution, and the nanocrystal size, and thus nanocrystal growth can be controlled by considering these parameters. During the growth of CdSe particles, there is a thermodynamic equilibrium between the Cd and Se atoms assembled on the nanocrystal surface and the corresponding free species in solution. This means that Cd and Se atoms are continuously binding and unbinding to the particles surface, and consequently the nanocrystals, at a given time, are either growing or dissolving. One important parameter to consider in the growth process is the surface-to-volume ratio of the nanocrystals, which dictates their overall reactivity. The smaller the nanocrystals, the higher their surface tension is. As a matter of fact, smaller nanocrystals are thermodynamically less stable than larger ones, because they have a higher ratio of surface to bulk atoms. In the presence of a high concentration of free atomic species in solution (a situation usually encountered at the early stages of the growth), smaller crystals grow faster than larger crystals, since they are more reactive and tend to incorporate the incoming free species more rapidly, which in turn lowers the ratio of surface to bulk atoms. In this case, the starting distribution of sizes narrows over time, a situation defined as a “focusing regime”. Over time, however, the concentration of free species in solution drops, and fresh monomers, which need to diffuse from the bulk of the solution to the surface of the growing nanocrystals, are not able to sustain the fast growth of the smaller particles. The growth rate of the large particles then becomes higher than that of the smaller particles, and the size distribution begins to broaden (the “defocusing regime”). Finally, when the concentration of free species in solution drops further, small particles, which are highly reactive, start dissolving and release free atomic species back into the solution. These species then feed the larger crystals, which are more stable, and the overall size distribution broadens much faster over time, a phenomenon known as “Ostwald ripening”.

Once the parameters affecting growth are well understood, manipulation of the growth kinetics can lead to the formation of nearly monodisperse nanocrystals of any desired size. In order to keep the system in the focusing regime for a long time, it is possible to nucleate just a few nanocrystals, so that they will compete less with the remaining free species in solution, which will then be depleted over a much longer timeframe. Alternatively, it is possible to perform additional slow injections of precursors during the growth, effectively keeping the concentration of free species in solution above a critical threshold. Based on these principles, high-quality spherical nanoparticles of a large variety of semiconductor materials, in addition to CdSe, are now produced routinely, such as CdS, CdTe,[27,28] InAs, InP,[29,30] and Ge.[31] Recently, the synthetic methods described above have been used to synthesize relatively monodisperse nanocrystals of oxides (such as Fe$_2$O$_3$,[32] Fe$_3$O$_4$, CoFe$_2$O$_4$, MnFe$_2$O$_4$,[33]) and of metals and metal alloys (such as FePt,[34] Co,[35–37] and CoPt,[38,39] see Figure 2).

Crystals are intrinsically anisotropic objects and the assumption that various nanocrystal facets will have similar surface energies under given reaction conditions is a rough approximation. In a crystal, different facets have different arrangements and densities of atoms, polarity, and number of dangling bonds. Consequently, the ability of a surfactant to coordinate surface atoms will vary face-by-face. In addition, a surfactant can be multidentate, and so its ability to bind to multiple surface atoms can be enhanced or limited by specific surface stereochemistry requirements. The combination of these factors leads to differences in surface energies and therefore in growth rates of the various facets. Frequently, the growth of colloidal nanocrystals is isotropic, which indicates that the influence of these factors is not so relevant. However, several research groups in the last few years have exploited this fact and have proposed various approaches aimed at synthesizing colloidal nanocrystals with anisotropic shapes. It is now possible to synthesize nanocrystals of various materials that have rod-like, disk-like, or even more complex shapes, such as the tetrapods.[30]
Recently, shape-controlled growth has been demonstrated for a wide range of materials, such as TiO$_2$\textsuperscript{[41]}, Co\textsuperscript{[42]}, CdSe\textsuperscript{[40,43]}, CdTe\textsuperscript{[44]}, ZnTe\textsuperscript{[45]}, and Ni\textsuperscript{[46]}. A detailed review about the shape-controlled growth of nanocrystals has been reported elsewhere\textsuperscript{[47]}.

At the next level of complexity, nanocrystals of one material can be embedded in a shell of another material, and in some cases epitaxial growth is even possible. For example, the growth of epitaxial shells of ZnS around spherical\textsuperscript{[48,49]} and rod-shaped CdSe nanoparticles is well established\textsuperscript{[50,51]}. Other examples for core/shell systems include CdSe/CdS\textsuperscript{[52,53]}, CdTe/CdSe and CdSe/ZnTe\textsuperscript{[54]}, InAs/InP and InAs/CdSe\textsuperscript{[55]}, FePt/Fe$_2$O$_3$\textsuperscript{[56]}, Pt/Co\textsuperscript{[57]}, and Ag/Co\textsuperscript{[58]}. In these cases the system as a whole is still highly symmetric, however, it is also possible to grow one material in an asymmetric fashion onto another one. Such heterostructures have been demonstrated for CdSe–CdS\textsuperscript{[59]}, CdS–FePt\textsuperscript{[60]}, and CdSe–Au\textsuperscript{[134]} (Figure 2).

Colloidal nanocrystals can possess a variety of physical properties. For example, Fe$_2$O$_3$ and CoPt$_3$ nanoparticles exhibit superparamagnetic behavior, while semiconductor nanocrystals have peculiar fluorescence properties\textsuperscript{[10]}. They have a continuous absorption spectrum, symmetric and narrow emission, and reduced photobleaching (Figure 3). Most important, the wavelength of fluorescence depends on the particle size\textsuperscript{[2]} In this way all fluorescence colors (wavelengths) spanning from the infrared to the ultraviolet can be obtained by selecting the appropriate semiconductor material and by tuning the particle size. Particle shape is also a key factor (in contrast to spherical CdSe nanoparticles, rod-shaped CdSe nanoparticles have a polarized emission).\textsuperscript{[61]}

By combining different materials in one entity nanoparticles with several properties, such as fluorescence and certain magnetic behavior, can be obtained\textsuperscript{[30]}. The synthesis of colloidal nanoparticles of different materials and shape is already highly advanced and synthesis protocols for many systems have been published. However, the synthetic creation of nanohybrid materials with controlled composition is still in its infancy and exciting future developments in this direction can be expected.

As already stated, the nanoparticles are coated with hydrophobic stabilizing molecules, which renders them insoluble in aqueous solution; this effect can be used to select nanoparticles by size. The principle of the so-called “size-selective precipitation” is easy to understand (Figure 4): If a polar solvent (such as methanol) is added to a solution of hydrophobically capped nanoparticles dissolved in an organic solvent (such as toluene), the nanoparticles start to precipitate. Since larger nanoparticles are dispersed in a less stable way than smaller ones, the larger nanoparticles precipitate first. Thus, a polar solvent can be added until the first nanoparticles begin to precipitate. The solution is then centrifuged, whereby the larger nanoparticles settle down as a pellet and the smaller ones stay as a supernatant in solution. The pellet can then be redissolved in the organic solvent so that this solution contains the fraction of the largest nanoparticles. This process can be repeated in an iterative way, so that fractions containing different nanoparticle sizes can be obtained. This procedure is very important to obtain homogeneous products. As a further example, it is possible that a synthesis of CdSe tetrapods may also generate CdSe spheres. By using size-selective precipitation, the smaller spheres can be separated from the larger tetrapods and a pure sample of tetrapods can be obtained\textsuperscript{[44]}.

Figure 3. The optical properties of colloidal CdSe nanocrystals strongly depend on their size. The smaller the nanoparticle, the more blue-shifted its fluorescence is. The absorption and emission spectra of four different samples of CdSe nanocrystals with different sizes are shown. The absorption spectra of the nanoparticles are continuous in the UV region and have a peak whose position is shifted to shorter wavelength as they reduce in size. The fluorescence emission of the nanoparticles is fairly symmetric and narrow (30–35 nm full width at half maximum) and peaks at a wavelength that is a few nanometers red-shifted compared to their absorption peak. Because of the continuous absorption spectrum in the UV region, all nanoparticles of different color can be excited at one single wavelength. One particularity of CdSe nanocrystals is their reduced tendency to photobleach compared to typical organic fluorophores (upper right: nanoparticles of different sizes in solution; lower right: fluorescence of these solutions under illumination with a hand-held UV lamp).
3. Transferring Nanoparticles into Aqueous Solution

In order to use hydrophobic nanoparticles for biological applications, they first have to be transferred into aqueous solution. Figure 5 provides a general scheme for the conversion of hydrophobic nanoparticles into hydrophilic species. Stabilizing molecules coordinate with the surface of the nanoparticle; this binding procedure, and the stabilizing molecule, has to be chosen carefully for every nanoparticle material. Thiols, for example, are known to bind well to gold surfaces,[62] whereas amines bind to cobalt surfaces.[63] Therefore, alkyl chains with either thiol or amino groups are appropriate stabilizing molecules for gold and cobalt nanoparticles, respectively. The alkyl chains point away from the particle surface (Figure 5a), which means that the inorganic nanoparticles cannot touch each other; they are stabilized by the steric repulsion of the hydrophobic chains. If nanoparticles of a highly concentrated solution are immobilized on a surface by evaporation of the solvent, they can form tightly packed arrays, which can be seen by TEM analysis (left column of Figure 2). However, the minimum distance between the surfaces of two inorganic cores is typically found to be smaller than two times the length of the stabilizing molecules, which means that the shells of organic stabilizing molecules can partially penetrate each other.[64,65]

In general, there are two strategies to stabilize nanoparticles in aqueous solution. The first is based on the introduction of charge. Nanoparticles of similar charge repel each other and thus aggregation is prevented. However, in electrolytic solution charges are screened by counterions and the electrostatic repulsion is weakened, which at sufficiently high salt concentrations finally can yield to particle aggregation. Thus, particle stabilization by electrostatic repulsion is only possible for moderate salt concentrations. Furthermore, the charge of stabilizing molecules depends on the pH value of the solution. For pH values above 5–6 carboxy (COOH) groups become negatively charged (COO⁻), whereas they are neutral for pH values below 5–6. Nanoparticles stabilized by the charge of carboxy groups are therefore only stable under neutral and alkaline pH conditions.

An alternative method of stabilization is through steric repulsion. Here, the ligand molecules form hydrophilic “brushes” around the nanoparticle surface. These brushes prevent inorganic particle cores from coming into contact. Typical molecules used for this purpose are polyethylene glycol (PEG)[66] and dextrane.[67] By these methods, nanoparticles can be made water-soluble by providing them with a hydrophilic coating, which is either charged or consists of polymer brushes. The next challenge is how to coat initially hydrophobic nanoparticles with such a hydrophilic shell.

The most straightforward way to achieve such coatings is via ligand exchange, where the hydrophobic stabilizing molecules are exchanged by hydrophilic ones.[66–70] Although this method is simple and direct, there are certain disadvantages involved with it. One site of the stabilizing molecule has to be able to strongly coordinate to the surface of the inorganic nanoparticle. This
site has to be individually chosen for every nanoparticle material. Therefore, no general procedure that works for all nanoparticle materials exists. In addition, there exists for many materials no site that strongly coordinates to the particle surface. For example, thiols (-SH) are typically used to bind to the surface of fluorescent CdSe/ZnS nanoparticles. The hydrophilic head group of such stabilizing molecules is often a carboxy (electrostatic stabilization) or a polyethylene glycol (steric stabilization) group. Since the binding affinity of thiols to ZnS surfaces is only moderate, these nanoparticles are not stable in aqueous solution for long periods.\[72\] Eventually the thiol–ZnS bonds are broken and the stabilizing molecules are removed from the particle surface (this can occur either because the thiol groups are hydrolyzed or photo-oxidized). If, for example, mercaptospropionic acid (HS-CH\(_2\)-CH\(_2\)-COOH) stabilized CdSe/ZnS nanoparticles are dialyzed against an aqueous solution, the nanoparticles start to precipitate after some hours. This is because through dialysis there are no excess mercaptospropionic acid molecules in solution, and those molecules initially bound to the particle surface unbind after a certain period of time and cannot rebind. Although still frequently used, simple ligand exchange of the stabilizing molecules is not optimal for the water-solubilization of nanoparticles. Advanced performance can be obtained with stabilizing molecules that have more than one site that coordinates to the inorganic particle surface.\[73,74,135\]

More elaborate protocols are still based on ligand exchange of the stabilizing molecules, but with the possibility of cross-linking the shell of the stabilizing molecules. Even if the bond between one stabilizing molecule and the particle surface breaks, this molecule is still kept in place by cross-linking to neighboring stabilizing molecules. Again the hydrophilic part of the stabilizing molecules can be either charged or consist of a polymer brush. An advanced method using such a protocol is surface silanization: First, the original hydrophobic ligand shell is replaced by a layer of silane molecules. On one of their end groups, the silane molecules are modified with a group that binds to the particle surface, such as thiol groups to bind to ZnS surfaces. Silane molecules include silanol groups, which can cross-link through the formation of siloxane bonds. In this way, stabilizing shells with more than one layer can also be created. Added silane molecules are incorporated in such a layer under the formation of siloxane bonds. When the outer shell consists of hydrophilic silanes (either with a charged or polymer-based tail group), the resulting nanoparticles are stable in water. Surface silanization has been successfully employed for many different nanoparticle materials.\[63,66,75–80\] The use of cross-linked ligand shells improves water stability, however, ligand exchange is still involved, and therefore the silane molecules that bind to certain particle surfaces have to be chosen individually.

In contrast to the two methods described above, there are further strategies that do not involve ligand exchange. These methods are based on the addition of an extra layer around the original hydrophobic layer of stabilizing molecules. The additional layer can be stabilized by hydrophobic interactions: The hydrophobic part of amphiphilic molecules coordinates to the hydrophobic stabilizing shell around the nanoparticles, whereas their hydrophilic part points outwards into the solution, and thus facilitates water solubility. This concept can be used to form water-soluble hydrophobic nanoparticles in a very general way and has been already applied to membrane proteins and different types of nanoparticles (see Figure 6).\[82–84\] The great advantage of this concept is that it does not involve ligand exchange.

Therefore, the same procedure can be applied to almost any hydrophobically capped particle, regardless of the material of the inorganic core. However since multiple shells are involved, the overall size of the nanoparticles will certainly be larger in comparison with nanoparticles solubilized by direct exchange to a hydrophilic monolayer shell. On the other hand, a large variety of amphiphilic polymers can be used, which allows for the direct incorporation of different functionalities directly into the shell. These functionalities can then be used to link the nanocrystals to biological molecules.

4. DNA–Nanoparticle Conjugates

Many groups\[85,86\] have reported the attachment of biological molecules to water-soluble nanocrystals. From the conceptual point of view there are two possibilities: First, biological molecules that are modified with a chemical group that is reactive towards the nanoparticle surface can be directly attached to the nanoparticle surface. This involves a ligand exchange in which part of the stabilizing molecules are replaced by the biological molecules. For example, biological molecules with thiol groups can react with the surface of gold and CdSe/ZnS nanocrystals by
Several types of interaction can be used to attach biological molecules to nanoparticles. In the most primitive case the molecules are simply adsorbed either directly to the nanoparticle surface or to the shell of stabilizing molecules around the nanoparticles.[91,92] Better stability can be obtained through electrostatic interactions between biological molecules that are oppositely charged to the nanoparticles.[73,93] The most elegant method, however, is via the formation of chemical bonds between the biological molecules and the stabilizing shell around the nanoparticles.[18,66,86]

Frequently the stabilization shell is composed of thiol-modified oligonucleotides, which are strongly bound to the Au surface via the thiol group. This standard conjugation of Au nanoparticles with oligonucleotides, it is straightforward to simply add thiol-modified oligonucleotides to a solution of phosphine-stabilized nanoparticles. The oligonucleotides will partly replace the phosphine-stabilizing shell and bind strongly to the Au surface via the thiol group. This standard procedure has also routinely been employed to functionalize citrate-stabilized Au nanoparticles with oligonucleotides.[107]

From a conceptual viewpoint, it would be ideal to control the number of oligonucleotides attached per nanoparticle. This is of particular importance for the construction of DNA-mediated particle groupings. Let us assume that a DNA-mediated nanoparticle trimer is desired, in which two small Au nanoparticles should be attached to one large Au nanoparticle. For this purpose, a large nanoparticle with exactly two strands of DNA, and two small nanoparticles with exactly one strand of complementary DNA is required. If the large Au particle had three strands of DNA attached to which three small Au nanoparticles could bind, a particle tetramer would be obtained instead. Therefore, for the creation of defined building blocks the number of DNA molecules per particle has to be controlled. Unfortunately no easy direct synthesis to obtain nanoparticles with an exact DNA-molecule-to-particle ratio exists; if thiol-modified DNA and nanoparticles are mixed in a 1:1 ratio, a stoichiometric distribution will always be obtained. This means that besides nanoparticles with one DNA unit per particle, the reaction mixture will also contain nanoparticles without DNA and nanoparticles with two DNA molecules per particle. For this reason a method has to be found to extract the Au nanoparticles with the desired number of attached DNA molecules from the reaction mixture.

One possibility to achieve this goal is through the use of gel electrophoresis. A gel is basically a porous matrix of a polymer to which an electric voltage can be applied. Charged nanoparticles can move through the electric field within the gel, and their speed of migration depends on the degree of charge associated with the nanoparticle (the higher the charge, the more rapid the migration). The polymer matrix hinders the flux of the nanoparticles; the larger the nanoparticles, the harder for them it is to squeeze through the pores and the slower the speed of migration will be. Thus, gel electrophoresis is sensitive to both particle charge and size. Both phosphine-stabilized Au nanoparticles and DNA are negatively charged. It can therefore be assumed that the total change in surface charge density of the Au nanoparticles upon conjugation with DNA is of minor importance. On the other hand, the attachment of DNA certainly increases the total size of the Au–DNA conjugate: the more DNA that is attached, the larger the total diameter of the conjugate becomes. This assumption corresponds well with our experimental findings.[87] Conjugation of nanoparticles with DNA decreases the speed of migration on the gel, which corresponds to an increase in conjugate diameter. The addition of charge, on the other hand, should increase the speed of migration, which cannot be observed in experiments. Thus, gel electrophoresis can be employed to sort DNA–Au conjugates by their size, and in parallel, by the number of DNA molecules attached per particle.[100] An example of such discrimination is shown in Figure 7b. Indeed, discrete bands corresponding to nanoparticles with zero, one, two, three, and four DNA molecules per Au particle can be resolved. The DNA–particle conjugates can be extracted from these bands of the gel[71] and used for further experiments. A similar gel-sorting concept has also been applied for different types of nanoparticles.[101] Unfortunately gel-sorting requires very good size and charge distributions of the unconjugated nanoparticles to afford good resolution of discrete bands.[86]

It should be noted that sorting by charge is also possible through gel electrophoresis techniques. For uncharged unconjugated nanoparticles, the conjugation with DNA primarily increases the surface-charge density. Therefore, these conjugates migrate faster as more DNA is attached.[96] Recently more direct concepts for the creation of nanoparticles with a controlled number of attached biological molecules have been reported that do not require gel sorting.[102] Once DNA–particle conjugates with a controlled number of DNA molecules per particle are available, DNA-mediated particle groupings can be formed. For the creation of particle dimers, two types of nanoparticles each bearing complementary strands of oligonucleotides are required. When both DNA–particle conjugates are brought together under suitable buffer conditions the complementary strands of DNA start to hybridize and thus link the two nanoparticles together. So far this method has been successfully employed for the creation of DNA-mediated dimers and trimers of Au nanoparticles (Figure 7c).[11] To date, no larger structures with controlled composition have been reported.
This is mainly due to the fact that the hybridization efficiency of DNA attached to Au nanoparticles is impaired. Part of the oligonucleotide can bind to the particle surface in a nonspecific way and is therefore only partly accessible for hybridization.

For some systems the number of attached DNA molecules per particle is yet to be controlled. However, such systems provide many possible applications, mainly in the area of biosensing. The group of Mirkin has developed very elaborate protocols to use DNA-modified Au nanoparticles (with many DNA molecules per particle) for the detection of DNA-sequences.\[103,104\] By using aptamer sequences as DNA, even molecules other than DNA can be detected with this universal method.\[105\] In addition, DNA-modified semiconductor nanoparticles have been used as fluorescent labels for DNA chips.\[14\]

So far, DNA–particle conjugates either with a small, discrete, and controllable number of DNA molecules per particle have been discussed as well as those with an uncontrollable number of DNA molecules per particle. However, there must be a limit to the number of DNA molecules that can be attached, a point where the particle surface will be completely saturated with DNA. This situation can be observed very well with gel electrophoresis. Once the Au particle is completely loaded with DNA the speed of migration reaches saturation.\[87\] In the case of 13 and 15.7-nm-diameter Au nanoparticles, a maximum of 115 and 157 single-stranded oligonucleotides of 12 bases could be bound per nanoparticle, respectively.\[106,107\] Further, it could be shown that the short oligonucleotides are fully stretched.\[87\] Upon the addition of more and more DNA the effective particle diameter becomes larger and larger; for steric reasons, when the particle is fully loaded with DNA the oligonucleotides point outward, fully extended along its contour length (see Figure 8a). Therefore, the total conjugate diameter is two
times the contour length of the DNA molecules plus the diameter of the Au particle.

The ability to fully stretch short DNA molecules provides the possibility of creating a spacer on the nanometer scale. One interesting photophysical problem is the quenching of fluorescence in the vicinity of gold surfaces. For detailed studies of this phenomenon, it would be desirable to precisely control the distance between the fluorescence dye and the gold surface. DNA-saturated Au nanoparticles seem to be a promising system for such studies. The fluorescent dye could be covalently attached on one end of the oligonucleotide, whose other end would be modified with a thiol group to bind to the gold surface. By using gel electrophoresis the saturation of the Au nanoparticles with DNA can be controlled. Since the DNA is fully stretched at full saturation, the distance between the fluorescence dye and the Au surface would be the contour length of the oligonucleotide (Figure 8b). By using DNA of different lengths, separations between 1 and 10 nm could be generated,[87] a respective study is under way.

Although this report is limited to the modification of Au nanoparticles with DNA, other particle materials and other biological molecules have also been used. In the future, such systems will allow for the controlled production of hybrid materials on the nanometer scale; many reports in these fields are predicted.

5. Nanoparticle Uptake by Living Cells

Colloidal semiconductor nanocrystals can emit fluorescence, which is suggestive of their use for the fluorescence labeling of cells. Compared to organic fluorophores they suffer less from photobleaching and easily allow for multiplexing (one excitation source, many channels of different colors). Soon after high-quality nanocrystals could be transferred to aqueous solution, the first labeling experiments of cells were reported.[16,86] These experiments were based on the conjugation of nanocrystals with biological ligands that specifically bind against certain cellular structures or compartments. In one of the original papers, CdSe/ZnS nanocrystals were modified with phalloidin, which triggered specific binding of these conjugates to the actin network of fibroblasts.[86] Since then, the multicolor labeling of different structures of fixed and living cells has been reported.[17,18] Recently, nanoparticles have been modified with biological molecules to facilitate the binding of conjugates to membrane-bound receptors.[87]

Because of reduced photobleaching the diffusion pathway of the receptor molecules within the cell membrane could be recorded over extended periods of time. It is predicted that the main labeling applications of fluorescent nanoparticles will be found in this field of single-molecule tracing. For a more detailed overview about labeling experiments with nanocrystals we refer the reader to already published reviews.[106-111]

In this review, the focus is towards another direction. Besides the labeling of cellular structures it has been also observed that living cells ingest colloidal nanocrystals. If cells are exposed to a culture medium that contains nanocrystals, they start to uptake the nanoparticles by endocytosis.[86,112-115] Nanoparticles are then transported to vesicular compartments around the nucleus of the cell where they remain.[91,112-115] Some authors have claimed receptor-mediated uptake,[97,114] whereas others report nonspecific uptake. As mentioned above there are several ways to transfer nanoparticles into aqueous solution, and certainly the ingestion of the nanoparticles might well depend on the particular protocol. In Figure 9 the distribution of CdSe/ZnS nanoparticles that were incorporated into a cell is shown. The effect of particle uptake can be used to label cells and follow their pathway or fate. Let us assume the following situation: Two different types of cells have to be cultured in parallel. With phase-contrast microscopy it is often not possible to determine the type of a cell, since the shapes of many cells can vary dramatically. Certainly, cells of one type can be stained specifically with a fluorescence-labeled antibody, which only binds to this particular type of cell. However, such staining impairs the cells and is thus not appropriate for following the fate of individual cells. Labeling with nanocrystals could be an interesting alternative. If cells of type A are incubated with green fluorescent nanoparticles, and cells of type B in another flask with red fluorescent nanoparticles, the cells ingest the respective nanoparticles. Thus cells of type A show green fluorescence and cells of type B exhibit red fluorescence. If both types of cells are finally seeded in co-culture on the same substrate, each type of cell can be identified by its color of fluorescence (Figure 10). Nanocrystals are suitable labels for several reasons: First, they have a reduced tendency to photobleach. Second, they are relatively biocompatible.[114] Third, upon cell division the nanoparticles are passed to both daughter cells and therefore the label is not lost.[91,112,116,118] These facts facilitate long-term observation of the fate of individual cells. With this technique the fate of a subpopulation of dictyostelium cells within a whole population could be traced.[118] In another experiment nanocrystals were microinjected into...
specific cells of Xenopus embryos to follow their development.\textsuperscript{[118]} Due to their particular properties, an increased use of fluorescent nanoparticles in this kind of fate-mapping study is expected.

Alternatively, the fate of individual cells on a cell culture substrate can be followed by using the so-called phagokinetic track method;\textsuperscript{[119–121]} the principle is illustrated in Figure 11. Let us assume a cell culture substrate is homogeneously coated with red fluorescent nanoparticles. Under the fluorescence microscope the substrate will appear simply red. Cells will then be cultured on the nanoparticle-coated substrate; the cells will start to ingest the nanoparticles. Compared to nanoparticles dissolved in solution the uptake of nanoparticles adsorbed to a surface is more efficient. The issue of three-dimensional diffusion of a dissolved particle into a cell now is reduced to a two-dimensional one for adsorbed nanoparticles. Adherent cells can migrate along the culture substrate. Wherever the cell moves, it ingests the nanoparticles of the underlying particle layer and stores the nanoparticles around its nucleus. This means that along the pathway of every cell the particle layer has been removed. Due to this phenomenon, the migration pathway of every cell appears dark under the fluorescence microscope, whereas areas where no cells have passed still exhibit homogeneous fluorescence. The fluorescence image of the culture substrate thus resembles a blueprint of the migration pathway of the cells.\textsuperscript{[112]} The speed and pattern of migration differs from cell type to cell type.

The phagokinetic track technique is a convenient method to determine the migration properties of cells. The more cells migrate, the larger the area cleared of nanocrystals per cell will be. This procedure could be highly automated; with fluorescence microscopy, intensity levels can be observed. One can observe the homogenous fluorescence of the undistorted layer, enhanced fluorescence within the cells that have accumulated the nanoparticles, and no fluorescence at positions that have been traversed by cells. The nonfluorescent area around each cell is characteristic for different types of cells.\textsuperscript{[122]} It has been demonstrated that for different cell types derived from breast tissue the area cleared of nanoparticles per cell is higher where the metastatic potential of the cells is at its highest.\textsuperscript{[122]} This might be the basis of a test for the metastatic potential of living cells.

Figure 10. MDA-MB-435s and MCF-7 cells were fed with green and red fluorescent silica-coated CdSe/ZnS nanocrystals, respectively, in separate culture flasks. Both types of cells were then mixed and seeded on the same substrate. The overlay of the phase contrast and fluorescence images is shown after some hours of incubation. Each cell can now be identified by the color of its fluorescence.

Figure 11. Phagokinetic tracks: a) Cells (gray) seeded on top of a collagen layer mixed with fluorescent nanocrystals (red), start to ingest the nanocrystals while they crawl along the layer (white arrows indicate the direction of cell movement). The ingested nanocrystals are stored inside the cells (red spot inside the cell). Areas of the layer that have been cleared of nanocrystals no longer fluoresce (black areas). The longer the incubation time, the more the cells move and the larger the non-fluorescent areas become;\textsuperscript{[122]} b–d) phase-contrast, fluorescence, and overlay images of a time series of phagokinetic tracks; the phase-contrast image shows the cell, while the fluorescence image displays the nanocrystal layer. The images were recorded for MCF-7 breast cancer cells seeded on top of a collagen layer infiltrated with silica-coated red fluorescent CdSe/ZnS nanocrystals.\textsuperscript{[112,122]} The images were recorded 30–130 min after seeding the cells on the layer; the hole in the layer clearly becomes larger with time. The fluorescence of nanocrystals accumulated inside the cells cannot be seen in these fluorescence images because the focus of the microscope was adjusted to the layer surface and not on the middle of the cells.
6. Outlook

In this review, we have described some biological applications of colloidal nanocrystals, in particular of fluorescent semiconductor nanoparticles. Besides labeling applications, nanocrystals have also been used for other purposes. The use of magnetic nanoparticles in drug delivery is particularly worthy of mention.\textsuperscript{[123]} Drugs can be immobilized on the surface of magnetic nanocrystals and the resulting drug–nanocrystal assemblies can be directed with magnetic fields to the target tissue.\textsuperscript{[124]} Typically, however, each particle material exhibits only one “feature”: Semiconductor nanoparticles are fluorescent and some metal and metal oxide nanoparticles are magnetic. For many applications, it would be desirable to have a material that combined these properties. The combination of a fluorescent and a magnetic particle to one material would allow for visualization by fluorescence and manipulation in magnetic fields. It is therefore proposed that the creation of materials on the nanometer scale with several properties will have a large future. The following section discusses several strategies as to how such nanohybrid materials could be obtained (see Figure 12).

Nanohybrid particles could be directly obtained through chemical synthesis by selectively growing one material on top of certain positions of another. The growth of ZnS shells around CdSe cores was demonstrated several years ago.\textsuperscript{[48]} Nowadays fluorescent nanoparticles can also be directly grown on to magnetic ones.\textsuperscript{[60]} This is an excellent demonstration of the combination of two properties in one particle. The clear advantage of this method is that when nanoparticles are grown on top of each other there is a negligible space between the domains. This should facilitate experiments in which the coupling between the different domains in hybrid nanoparticles is to be investigated. No energy transfer or tunneling is possible when nanoparticles are further apart than a few nanometers. Also the connection between the individual domains should be very stable. However, the large disadvantage of this method is that it will almost certainly be limited to relatively primitive structures. One could, for example, imagine spheres of one material grown on the ends of a tetrapod shaped nanoparticle of another material. However, it does not seem feasible to grow more than a few materials in one hybrid system.

Larger structures could be obtained through linkage with biological molecules. Almost 10 years ago, the first examples of DNA-mediated particle assemblies were reported.\textsuperscript{[85,88,125]} However, the obtained assemblies were more like aggregates than well-defined structures. Today, much effort is invested in the assembly of exactly defined structures. Such examples have already been demonstrated with the construction of DNA-mediated particle groupings.\textsuperscript{[6,7]} This method in principle would allow for the construction of complex structures composed of multiple nanoparticles of different materials. Some work in this direction has already been reported.\textsuperscript{[126]} The use of DNA as a linker offers the ability to precisely program the linkage by selecting appropriate sequences of complementary oligonucleotides.

Unfortunately there are also some conceptual disadvantages involved with this technique. First, the biological molecules that are employed to link the nanoparticles act as a spacer between the nanoparticles. Thus, there always will be a gap between the individual nanoparticles within one particular grouping. Second, the biological molecules and especially their linkage to the nanoparticles are not stiff, but rather flexible. Such flexibility suggests that the nanoparticles will not be linked in a rigid assembly, but rather the whole construct

![Figure 12. One goal of nanotechnology is the construction of materials with new properties, for example the combination of magnetic and fluorescent properties in one material. Hybrid structures comprising three different materials are systematically sketched (drawn in green, red, and blue). a) Different materials can be grown directly on each other. This elegant and stable method will be limited to small structures (the growth of Au nanoparticles at the end of CdSe rods is shown; image courtesy of U. Banin et al.\textsuperscript{[134]}); b) nanoparticles of different materials can be grown separately and then linked by biological molecules. These systems will be more general, but less stable because the linker-molecules keep individual nanoparticles further apart (the DNA-mediated linkage of Au nanoparticles is shown; image courtesy of D. Zanchet et al.\textsuperscript{[27]}); c) a straightforward approach is to load containers with nanoparticles of different materials. Such hybrid systems are larger but much easier to prepare (polymer capsules containing fluorescent CdTe nanoparticles are shown; image courtesy of G. Sukhorukov, A. Rogach, and B. Zebli\textsuperscript{[126]}).](image)
will be flexible and the distance between the nanoparticles within the assembly will fluctuate. For example, a three-dimensional construct in solution might collapse to two dimensions once it is adsorbed and dried on a surface. Third, the stability of the linkage is limited. Biological molecules are bound by certain conditions. DNA-mediated particle groupings certainly will not be able to withstand high temperatures (≥100°C) at which biological molecules start to degrade. There also exists possibilities of destruction in solution. For example, enzymes might digest part of the linker molecules. With DNA the linkage can also be broken by increasing the temperature or by decreasing the salt concentration, that is, by reducing the melting temperature. Although these factors limit stability, they could also be used for the controlled release of nanoparticles from the particle assembly.

Maybe the easiest way to combine nanoparticles of different functionality is to simply pack them in one container. Such an assembly will typically have dimensions on the microscale rather than the nanoscale, but certainly they would offer the possibility of combining as many different materials as desired. One example in this direction is the incorporation of nanoparticles in hollow polymer capsules. Such capsules are assembled layer by layer.\[12,126\] First a charged template, for example a microsphere, is required. Then polymer molecules of opposite charge are added that bind electrostatically to the template. Unbound excess polymer is removed, then a polymer of opposite charge to the first polymer layer is added, which adsorbs by electrostatic interaction. The excess polymer is then removed. These steps are iterated and a multilayer construct like an onion of many successive polymer layers of opposite charge is formed. Finally the template is dissolved and a hollow sphere composed of polyelectrolyte multilayers is obtained. Nanoparticles can be introduced in two different ways. By controlling the pH- and salt conditions the mesh size of the capsule walls can be controlled. In this way the nanoparticles can be introduced since the capsule walls are permeable, and can then be trapped inside the capsule by rendering the capsule wall impermeable. Alternatively, nanoparticles can be incorporated into the capsule walls. When the outer layer of the capsules is positively charged, then negatively charged nanoparticles will adsorb to this layer. Unbound nanoparticles are removed and positively charged polymer is added. In this way nanoparticles of different functionality can be incorporated at different layers of the capsule wall. The groups of Sukhorukov and Rogach have demonstrated the construction of magnetic and fluorescent capsules by incorporating magnetic and fluorescent nanoparticles to the capsule walls.\[29\] Such constructs hold great promise in many biological applications, such as drug delivery.\[290\]

To conclude this Review, it should be pointed out that the synthesis of colloidal nanocrystals and their modification with biological molecules is already highly advanced. Research on such nanoparticles has changed from development and characterization to real applications. Nevertheless, it is predicted that efforts in particle synthesis will shift towards the construction of heterostructured nanoparticles that combine multiple functionalities.

Acknowledgement

This project was supported by the Deutsche Forschungsgemeinschaft (DFG, Emmy Noether grant WP), by the Deutscher Akademischer Austauschdienst (DAAD, Vigoni Project), and by the Fonds der Chemischen Industrie. The authors are grateful to Prof. U. Banin and Drs. G. Sukhorukov and A. Rogach for providing images of their work for this Review. Many of the ideas described in this Review are based on work initiated in the group of Prof. A. P. Alivisatos, in whose groups some of the authors have worked previously.


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