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Biofunctionalization of Fluorescent Nanoparticles

Michael J. Murcia and Christoph A. Naumann

1.1
Introduction

The current revolution in life sciences is strongly linked to the availability of sophisticated new experimental tools that enable the manipulation of biomolecules and the study of biological processes at the molecular level using state-of-the-art imaging techniques, such as single molecule imaging. Optical microscopy is fundamental to furthering our understanding of the structural, organizational, and dynamic properties of biological systems because a wide variety of complementary, non-invasive optical techniques exemplified by wide-field microscopy techniques, such as brightfield, darkfield, phase contrast, and DIC exist. Among these optical detection techniques, fluorescence microscopy is particularly important because it facilitates highly sensitive and specific imaging experiments. In addition, more sophisticated imaging approaches such as confocal and near-field imaging provide the opportunity for 3D and sub-diffraction limit imaging, respectively.

Optical microscopy is now sensitive enough to track individual molecules if they are conjugated to appropriate imaging probes. Traditionally, such single molecule probes were μm-size colloidal particles and single fluorophores [1]. Colloidal probes such as gold or fluorescently labeled polystyrene beads are typically much larger (0.1–1 μm) than the biomolecule to be studied. However, fluorescent dyes, though smaller, show pronounced photo-instabilities, including blinking (due to fluorescence intensity fluctuations) and photobleaching, thus complicating single molecule tracking experiments and other fluorescence-based long-term studies. From the above description, further progress in the field of optical single molecule imaging obviously depends on the availability of appropriate labels that combine small size and high photostability with the ability to be used in multicolor studies. Fluorescent nanoparticles fulfill these important criteria. To be used in a biological environment, these nanoprobes need to be biofunctionalized appropriately, which remains a significant challenge.

The main focus of this chapter is to provide an overview of recent developments addressing the bioconjugation of fluorescent nanoparticles and their surface modification using biocompatible coatings. Section 1.2 summarizes the different types

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of fluorescent nanoparticles, including dye-doped nanoparticles, quantum dots (QDs), metal nanoparticles, and μm-size hybrids comprising fluorescent nanoprobes. Section 1.3 describes recent developments concerning the conjugation of biomolecules to fluorescent nanoparticles, and compares the different conjugation approaches for nanoparticles consisting of polymeric, semiconductor, or metallic materials. An overview of published design architectures of biocompatible surface coatings as applied to fluorescent nanoparticles is given in Section 1.4. Finally, Section 1.5 lists representative examples of how biofunctionalized nanoprobes can be applied to problems in biosensing, as well as single cell and biological tissue imaging.

1.2 Fluorescent Nanoparticle Probes

Gold nanoparticles that are surface-functionalized with proteins have been used in electron microscopy (EM) applications for quite some time [2]. A prominent example is the specific labeling of tissue by use of antibody-conjugated Au-nanoprobes (10–40 nm diameter) and their imaging by use of transmission electron microscopy (TEM). Such EM studies can achieve the detection of Au-probes with a resolution of less than 10 nm [3]. Though this demonstrates a great sensitivity, EM is limited by its inability to image living biological systems. Optical microscopy may overcome this limitation. For example, colloidal gold of 30–40 nm diameter has been used as an optical imaging label on multiple single molecule tracking experiments at the cellular level [1, and references therein]. Here, colloidal gold is used as a Rayleigh scatterer, for which the scattering $\propto d^6$ (d = diameter), thus making tracking experiments with probe diameters of less than 30 nm extremely challenging or even impossible.

Fluorescent nanoparticles are highly attractive imaging probes because, in contrast to scattering probes, their detection is not limited by the Rayleigh scattering condition. As a consequence, fluorescent nanoprobes > 1 nm can be detected if appropriate imaging setups are used. Importantly, at this size range, such nanoprobes do not exceed the size of individual proteins, thus addressing an important condition for high-quality imaging at the molecular level. In addition, single molecule detection is improved because the weak scattering of the probes lowers the optical background and thus enhances the imaging sensitivity. Figure 1.1 illustrates the size range of these probes in comparison with that of other nanoparticles. The most common types of fluorescent nanoparticles, dye-doped nanospheres and luminescent quantum dots (QDs), are described below in more detail, together with some recently introduced hybrid architectures of fluorescent nanoprobe-containing μm-size particles. Also included is an overview of optically active metal nanoparticles because these probes are highly relevant in single molecule spectroscopy.

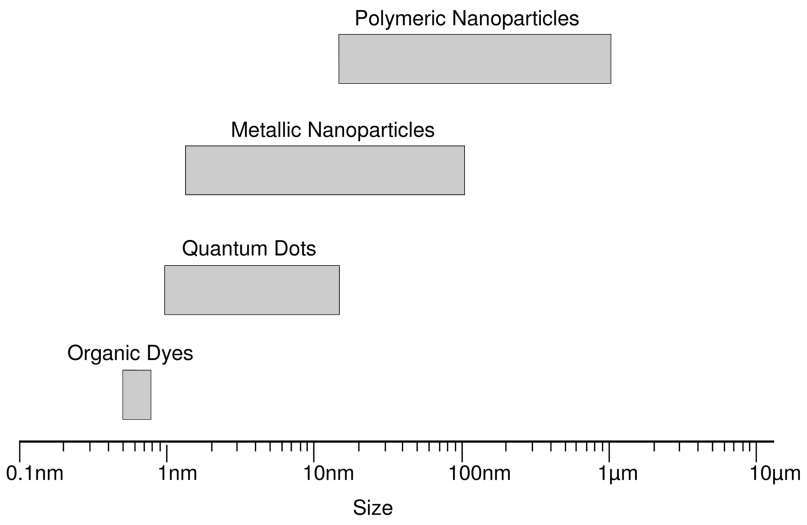


Fig. 1.1. Size ranges of commonly used fluorescent nanoprobes.

1.2.1 Dye-doped Nanoparticles

Dye-doped nanoparticles are polymer or silica-based particles containing organic or inorganic dyes [4, 5]. Dyes can be attached to the nanoparticle surface or can be embedded inside the particles either noncovalently or covalently. For imaging applications, dye-doped nanoparticles containing embedded dyes are particularly attractive because their photostability can be enhanced due to the better protection of the dyes from oxygen. For example, incorporation of pyrene dyes into polystyrene particles using a normal microemulsion approach led to a 40-fold increase in emission intensity with respect to the pure dye at the identical concentration [6]. Brightness of the fluorescence signal from such imaging probes can be controlled by the number of dye molecules per nanoparticle, with the maximum dye density limited only by self-quenching. Therefore, dye-doped nanoparticles can be quite photostable without showing fluorescence intensity fluctuations (blinking).

Typical polymer-based dye-doped nanoparticles are made of hydrophobic polymers. The hydrophobic dye molecules are kept within such nanoparticles through noncovalent hydrophobic interactions, thus preventing the gradual release and photooxidation of the dyes. Dye-doped polymeric nanoparticles have been applied down to a size of ~20 nm [7]. Although they are reasonably photostable, the hydrophobic nature of these probes complicates imaging applications in aqueous environments, which are required for studies on biological systems. Common unwanted phenomena are clustering and non-specific binding. To overcome these problems, the hydrophobic core particles can be surface-functionalized with hydro-

philic coatings such as polymers like poly(ethylene glycol) (PEG), polysaccharides such as dextran, or proteins such as bovine serum albumin (BSA).

Dye-doped silica nanoparticles are attractive imaging probes because their hydrophilic nature reduces the problems of non-specific binding and clustering. Silica shows several additional properties beneficial for optical imaging applications in biological systems, including chemical inertness, transparency, and the ability to act as stabilizers in protecting the embedded dyes from the outside environment [4]. In addition, the surface hydroxyl groups can be chemically modified, allowing for the straightforward surface modification with amines, carboxyls, or thiols. In addition, pH changes do not lead to swelling and porosity changes, and silica particles are less prone to attack from microbes [8]. However, the incorporation of hydrophobic dyes into the hydrophilic silica matrix is challenging, requiring specific modifications of either the dye molecules or the silica. For example, dyes can be modified with a hydrophilic molecule such as dextran or a hydrophobic silica-precursor can be used during nanoparticle synthesis [9]. Dye-doped silica particles were first synthesized using the Stoeber method [10]. However, this method leads to polydispersity and average particle sizes of >100 nm. More recently, these limitations have been overcome by use of a reverse microemulsion method that can create monodisperse dye-doped silica particles down to a size of 15 nm (Fig. 1.2) [11]. Notably, this elegant approach facilitates the size-tuning via adjustment of the microemulsion composition and does not require elevated temperatures and pressures [11, 12]. Silica also has been used to create hollow silica nanospheres filled with dye molecules [13].

Rare-earth-doped $LaPO_4$ nanoparticles are another interesting class of nanoprobes that show promising properties for diagnostic and imaging applications [14]. These systems combine small size (<10 nm), high chemical stability, very good quantum yield, and high photostability. Furthermore, they are expected to show low cytotoxicity. Recently, such rare-earth-doped $LaPO_4$ nanoparticles were successfully surface-functionalized to allow their subsequent coupling to biomole-

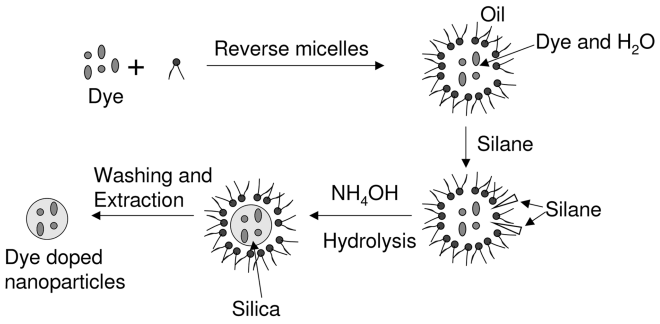


Fig. 1.2. Schematic of the reverse microemulsion method for the synthesis of dye-doped silica nanoparticles (adapted from Bagwe et al., 2004) [11]. In this method, nm-sized water droplets, which are stabilized by surfactant molecules, are formed in a continuous oil phase, thus forming a thermodynamically stable oil–water–surfactant microemulsion.

cules [15]. The nanoparticle surface was first functionalized with aminohexanoic acid and then linked to avidin using EDC coupling.

1.2.2 Quantum Dots (QDs)

Nanocrystals based on semiconductor materials began attracting the interest of physicists three decades ago because of their interesting quantum properties. These properties are the result of size-dependent band gaps, which cause the color emitted by a semiconductor nanocrystal of a specific composition to be a function of its diameter. Physically, quantum properties (in this case a size-dependent fluorescence emission) are expected to occur if electron–hole pairs (excitons) are confined to dimensions that are smaller than the electron–hole distance (exciton diameter) [16–19]. As a result of this condition, the state of free charge carriers within a nanocrystal is quantized and the spacing of the discrete energy states (emission colors) is linked to the size of the nanoparticle. It is this quantum confinement effect that led to the term ''quantum dot''.

Quantum dots can be based on metallic or semiconductor materials. Most widely used are CdSe and CdTe quantum dots because their quantum confinement region spans the entire optical spectrum. More recently, there has been growing interest in quantum dots with near-infrared emission properties, such as CdTe/CdSe, InAs, or PbS (which are of use in animal imaging studies) [20, and references therein]. In addition, several groups have studied silicon nanocrystals [21–23]. Quantum dots also show other fascinating optical properties, including broad absorption and narrow emission bands, which allows a single laser to excite dots of a wide size-range, with each dot emitting its own specific color. This is in contrast to organic-based fluorophores, which are characterized by narrow Stokes shifts (difference between maximum wavelengths of absorption and emission bands). To protect their surface from photobleaching, quantum dots can be passivated by use of a higher-bandgap semiconductor shell or an organic layer [24]. In fact, successfully passivated, quantum dots show dramatically enhanced photostability, enabling their long-term observation in optical experiments [25]. Furthermore, quantum dots can be brighter than their dye counterparts at an equivalent quantum yield [26] because of their notably higher extinction coefficients [20]. Given the above properties, quantum dots bring fascinating possibilities for single molecule cellular imaging studies because they combine small size, broad absorption, narrow size-tunable emission (covering the entire optical spectrum), and excellent photostability. These features outperform traditional fluorescent dyes in many respects.

The traditional approach to synthesizing quantum dots relies on heating specific organic solvents and injection of semiconductor precursors. In a typical preparation [27], $Cd(CH_3)_2$ and elemental Se are combined with trioctylphosphine oxide (TOPO), which acts as a solvent and stabilizing agent. This mixture is subjected to high temperature (about 350 °C) for 24 hours at which time the mixture is cooled and $Zn(CH_3)_2$ and $S(SiMe_3)_2$ added to form a stabilizing ZnS shell. To cre-

ate nanocrystals of a narrow size distribution, an additional size-selective precipitation step needs to be included. Because dialkylmetal compounds are very sensitive to oxygen and water and become pyrophoric upon exposure to air, alternative approaches of quantum dot synthesis have been explored. Consequently, CdSe quantum dots can be formed using CdO, selenium, and hexylphosphonic acid or tetradecylphosphonic acid [28]. This synthesis reduces the reaction times to less than 30 min, but still uses temperatures upwards of 300 °C. In another example, our group has synthesized CdSe/ZnS quantum dots by use of sonochemistry [29]. This low-temperature approach not only produces spherical high-quality quantum dots with quantum yields of up to 70% and emission bands of less than 50 nm (FWHM), but also allows for straightforward control of the synthesis parameters. Figure 1.3 shows the photoluminescence spectra of CdSe core quantum dots and

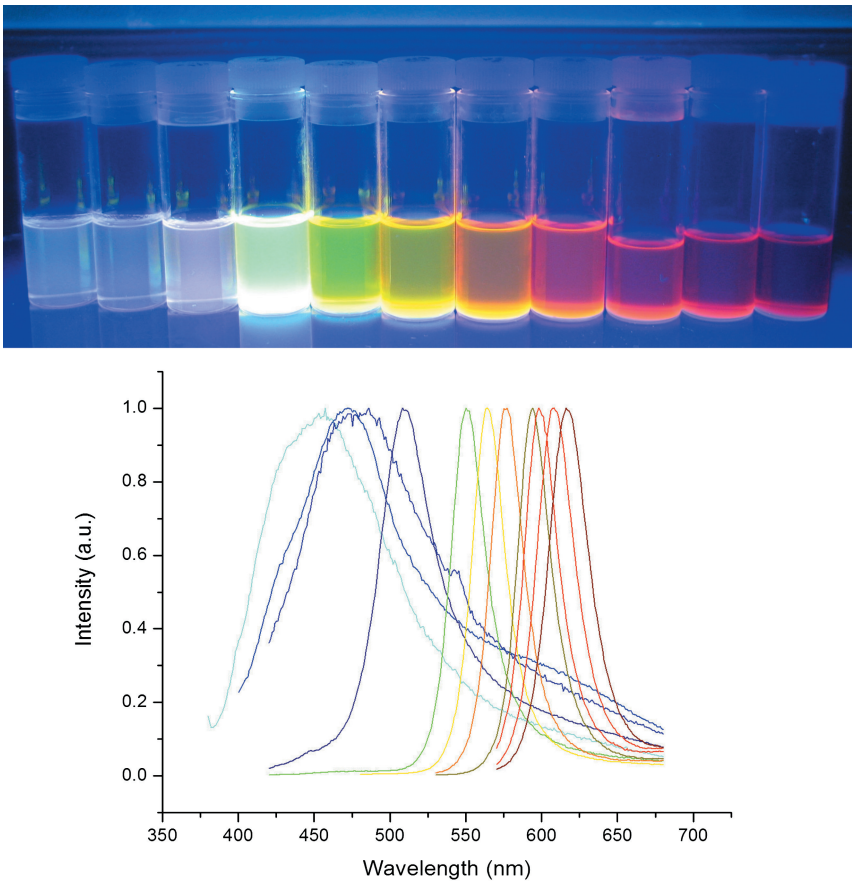


Fig. 1.3. Quantum dots show size-tunable emission properties. The samples represent different sizes of quantum dots, which produce different colors upon UV light. Within the quantum confinement region, an increase in particle sizes produces a redshift in the emission spectrum. Spectra and photograph were obtained from CdSe quantum dots synthesized sonochemically [29].

the colors of these samples upon UV illumination, which are representative of this procedure.

To be of use in biological imaging applications, quantum dots need to be water-soluble. Indeed, synthetic approaches for CdTe [30–32] and CdS [30, 33–38] have utilized aqueous solvent conditions. However, these nanocrystals usually lack the quantum yield and narrow size distribution observed for TOPO-synthesized quantum dots. TOPO-stabilized quantum dots, however, show hydrophobic surface properties. To disperse TOPO-stabilized quantum dots in aqueous solution, several surface modification strategies have been pursued (Fig. 1.4). A common approach is to synthesize quantum dots in TOPO and replace the hydrophobic TOPO layer with bifunctional molecules containing thiol and hydrophilic moieties separated by a molecular spacer (Fig. 1.4: approach I) [39, 40]. The thiol groups bind to the CdSe or ZnS surface, while the hydrophilic moieties radiate from the surface of the corresponding semiconductor. Unfortunately, thiols bind less strongly to ZnS than to Au, which leads to a dynamic equilibrium between bound and unbound thiols. This behavior reduces the long-term water solubility of ZnS-capped quantum dots. To shift the equilibrium towards bound moieties, monothiols have been replaced with molecules containing more than one thiol group (Fig. 1.4: approach II) [41–43]. Another stabilization concept is to enhance binding via surface cross-linking of bound molecules. On the basis of this concept, ZnS-shelled quantum dots have been made water-soluble by adding a silica shell to the nanoparticles by using alkoxysilanes during the polycondensation (Fig. 1.4: approach III) [44–48]. Two types of silanes have been used to stabilize quantum dots in aqueous solution. The first includes silanes whose surface functional groups are positively or negatively charged at neutral pH [48]. The second type includes silanes with poly(ethylene glycol) chains [48, 49]. TOPO-coatings also can be made water-soluble without their replacement by adding amphiphilic molecules such as lipopolymers or amphiphilic diblock copolymers, whose hydrophobic moiety stabilizes the TOPO-coating via hydrophobic forces and whose hydrophilic moiety is exposed to the solvent environment, guaranteeing water-solubility (Fig. 1.4: approach IV). The last approach has the advantage of not exposing the sensitive surface of the quantum dot during a surface exchange step.

1.2.3 Metal Nanoparticles

In contrast to noble metals in bulk, nanoparticular forms of such materials result in interesting photochemical and electronic properties [50]. Three strategies have been pursued to study the photochemical activity of metal nanoparticles: (a) direct excitation of the metal nanoparticles; (b) indirect excitation of the metal nanoparticles via surface-conjugated dye molecules; and (c) photocatalytic processes in semiconductor–metal nanocomposites [50]. After excitation with UV or visible light, metal nanoparticles show several interesting phenomena, including photoluminescence [51–53], nonlinear optical phenomena [54, 55], and surface-enhanced

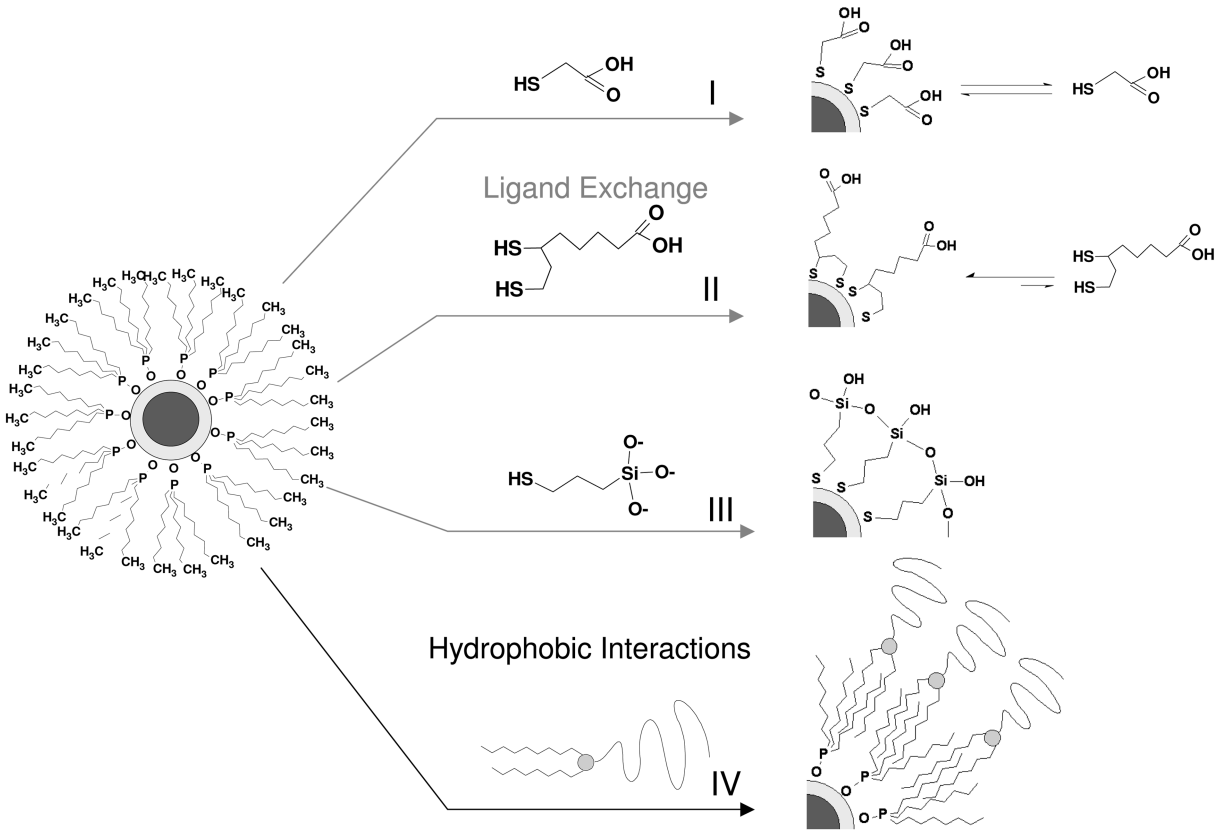


Fig. 1.4. Schematic representation of four common approaches to hydrophilic surface modification of TOPO-stabilized quantum dots. Approach I: TOPO replacement with a heterobifunctional linker consisting of a thiol end group, a spacer, and a hydrophilic end group such as carboxylic acid. II: TOPO replacement with a linker consisting of two thiol groups on one end and a hydrophilic end group on the other end. Approach III: TOPO replacement with a silane forming a stable shell via crosslinking. Approach IV: Stabilization of TOPO layer using amphiphilic molecules such as PEG lipopolymers or amphiphilic diblock copolymers that are held on the surface by hydrophobic interaction with the octyl chains of TOPO.

Raman scattering (SERS) [56–61]. Due to the significant field enhancement, SERS can be used as an extremely sensitive analytical tool, thereby exceeding the sensitivity from luminescence-detecting techniques. For example, biomolecules can be detected with 1000-fold better sensitivity if they are bound to Au nanoparticles [62]. Silver nanoparticles also are useful in this respect. The main experimental challenge in SERS is to keep the surface roughness uniform and reproducible.

There are multiple strategies for synthesizing metal nanoparticles [50]. For example, they can be synthesized using a biphasic reduction approach [63–69]. In this procedure, a noble metal salt such as $HAuCl_4$ is dissolved in water and phase-transfer extracted into an organic solvent followed by reduction with $NaBH_4$. Metal nanoparticles also have been synthesized using reverse micelle procedures where the size and size distribution of nanospheres can be controlled by the micelle composition [12, 70–73]. Gold nanoparticles are particularly attractive for studies in a biological environment because they show no surface oxidation and high biocompatibility without any surface modification. In addition, thiol chemistry can be applied to conjugate molecules to the gold surface.

1.2.4 Hybrid Architectures Involving Fluorescent Nanoprobes

1.2.4.1 Metal–Dye

Another interesting application of metal nanoparticles is their use in combination with conjugated dye molecules (Fig. 1.5). Such hybrid systems are attractive because they can be studied by use of electron microscopy in addition to fluorescence-based techniques such as fluorescence microscopy and spectroscopy. Importantly, if conjugated to biomolecules, metal nanoparticle–dye hybrids can be used as very sensitive biomolecular imaging probes [74, 75]. Interestingly, dye molecules attached to metal nanoparticles can show enhanced emission. For example, Py-CH_2NH_2 molecules bound to gold particles show pronounced emission that is much stronger than that for unbound Py-CH_2NH_2 in THF [50]. The main disadvantage of this approach is that the surface-exposure of the dyes promotes their photooxidation.

1.2.4.2 Dye-doped Silica Shells

To reduce photooxidation, dyes also can be embedded within the nanoparticle. As illustrated in Fig. 1.5(B), dyes can be incorporated into a nanoparticle-capping silica shell. For example, up to 12 alternating fluorescent and nonfluorescent silica shells have been added to silica core particles, where the shells were doped with six dyes of different emission wavelengths. Dyes of multiple colors have been incorporated previously in silica beads throughout the matrix of the particle [76], but not in an ordered fashion [77]. This multi-shell approach helps to reduce unwanted energy transfer. As a multiplexing probe, the system is not as simple to analyze as a quantum dot-based one. Re-absorption of the fluorescence emission,

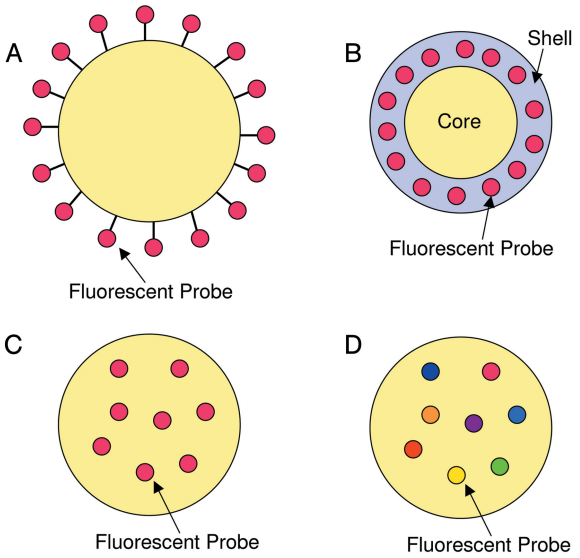


Fig. 1.5. Common hybrid architectures containing fluorescent nanoprobes include (A) dyes bound to a nanoparticle surface; (B) dyes incorporated into the silica shell of a nanoparticle; (C) fluorescent probes such quantum dots or dyes incorporated into the core of microparticles; and (D) a multiplexing approach by embedding probes of different colors.

which is related to the identity of the fluorophore, alters the emission intensity. As a result, it is difficult to determine the possible number of spectral combinations in a designed system, and a flow cytometer must be used to identify the number of individual species in the mixture. This shelling approach has also been reported for single dye systems for enhanced fluorescence [10].

1.2.4.3 **Quantum Dot-containing Microspheres**

An interesting hybrid architecture that has been developed recently is the quantum dot embedded microsphere. Unlike a dye embedded polymeric bead, the quantum dot microbeads utilize the narrow emission of quantum dots combined with their broad absorption characteristics to perform multicolor detection experiments with one excitation wavelength [78–81]. By incorporating up to six different colors of quantum dots and ten intensities of those colors, the quantum dot microbeads can, theoretically, optically "bar code" biomolecules with one million possible spectral signatures. Realistically, the number is likely to be substantially lower due to spectral overlap, fluorescence intensity variations, and signal-to-noise requirements. The multiplexing approach has been tried with organic dyes, but was limited to two colors due to spectral overlapping and the inability to excite more than two or three dyes with the same wavelength [82].

1.3
Bioconjugation of Fluorescent Nanoparticles

1.3.1
General Considerations

1.3.1.1 Overview

To apply fluorescent nanoparticles to biosensing and biomedical imaging applications, it is crucial to develop strategies towards their biofunctionalization. These include the proper linkage of biomolecules to nanoparticles (bioconjugation) and the design of appropriate biocompatible coatings. This section outlines the different aspects of bioconjugation, and Section 1.4 provides a corresponding discussion of biocompatible coatings.

Bioconjugation can be described as any procedure that links a nanoparticle to a biomolecule under mild conditions [83]. As described above, the synthesis of nanoparticles often does not render them capable of attachment to biomolecules, because their surface-chemical properties are not appropriate. Therefore, nanoparticles frequently must undergo surface transformations to create the chemistry needed for coupling to biomolecules under mild (physiological) conditions. There are a few key requirements for successful bioconjugation reactions [4]. Crucially, the conjugation process must avoid compromising the activity of biomolecules. In addition, the bioconjugation ideally should not hinder the signal of the nanoparticle. Another requirement is the ability to control the number of linkage sites on the nanoparticle surface where biomolecules can bind. This requirement can be quite challenging. In addition, the biomolecule–nanoparticle coupling should be stable and, for crystalline particles, the surface should be covered to avoid free valence states. Finally, the thickness of any nanoparticle shell should remain as small as possible relative to the nanoparticle size.

Figure 1.6 illustrates several possible strategies to bioconjugate nanoparticles. Simple adsorption of biomolecules to the nanoparticle surface via noncovalent forces represents the least demanding approach (Fig. 1.6A). However, in this case, the activity of bound biomolecules may be compromised and the amount of bound biomolecules per nanoparticle is difficult to control. In addition, this bioconjugation approach does not target one protein preferentially. Another concept is based on the physisorption (noncovalent coupling) of biomolecules to molecules (e.g., other biomolecules) acting as mediators between biomolecule and nanoparticle surface (Fig. 1.6B). This design may be advantageous over the first one, because it may help biomolecules to bind in a proper orientation. Figure 1.6(C) is based on the chemical coupling between reactive groups of biomolecules (e.g., thiols and primary amines) and crosslinker molecules. These crosslinkers may bind to the nanoparticle surface via physisorption or chemisorption (covalent coupling). However, there are frequently multiple active sites on the target biomolecule to which the probe can bind, thus preventing controlled binding. Furthermore, uncontrolled

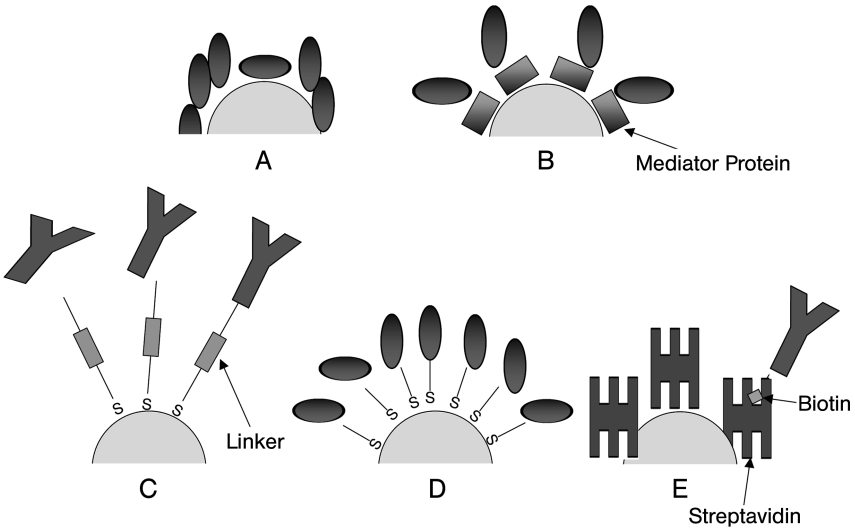


Fig. 1.6. Common strategies for the conjugation of biomolecules to nanoparticles include direct physisorption of biomolecules (A), assisted physisorption using pre-bound molecules (B), chemical linkage of biomolecules to crosslinkers either physisorbed or chemisorbed on the nanoparticle surface (C), direct chemical coupling of biomolecules to nanoparticles (D), and the targeted binding of biotinylated biomolecules to streptavidin-coated nanoparticles via biotin–streptavidin coupling (E).

binding may interfere with the biologically active sites of biomolecules. To overcome this limitation, a ligand (typically an antibody) can be bound to the nanoparticle via the crosslinker. This ligand then facilitates coupling to the biomolecule of interest with high specificity. These crosslinker-based bioconjugation strategies are best executed if appropriate heterobifunctional crosslinkers are used. A large variety of such crosslinker molecules is now commercially available. Alternatively, homobifunctional crosslinkers can be used where one reactive end group is protected. Here, nanoparticle and biomolecule are coupled by binding of the partially protected linker to either nanoparticle or biomolecule and by deprotecting the linker prior to bioconjugation. Unprotected homobifunctional crosslinkers are always problematic because they can induce the clustering of nanoparticles. Figure 1.6(D) shows a facile approach for the chemical coupling of biomolecules to nanoparticles. This approach is particularly useful for attaching oligonucleotides to nanoparticles, e.g., via mercapto groups [84–86]. Still, control over the number of bound biomolecules per nanoparticle remains a challenging endeavor. To achieve such control, a separation step, such as nanoparticle separation using gel electrophoresis, must be added [87]. Another popular approach is to link biotinylated ligands or target biomolecules to streptavidin (or avidin)-functionalized nanoparticles with high specificity (Fig. 1.6E). In this case, the biotin-binding proteins, avidin or streptavidin, act as linker molecules [88, 89].

1.3.1.2 Common Coupling Reactions

The bioconjugation of nanoparticles is critically dependent on the availability of effective covalent coupling procedures. Figure 1.7 lists the most common coupling procedures. The reaction between primary amine and carboxylic acid groups is a very popular coupling procedure (Fig. 1.7A). In a first step, a carboxylic acid reacts with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) and *N*-hydroxysulfosuccinimide (NHS) to form an acyl amino ester that subsequently reacts with the primary amine to create a stable amide bond. This approach also is attractive because preparation of the ligands is less demanding. A widely used approach is based on the reaction between thiol and maleimide groups (Fig. 1.7B). This affords a stable thioether bond, typically with good yield at physiological pH. Most prominently, this reaction can be used to directly link maleimide-functionalized ligands to thio-groups of proteins. If no thiols are available, they can be created by using heterobifunctional crosslinkers with one thiol endgroup or by reducing disulfide bonds within the target protein. The coupling of two thiols to form a disulfide linkage is another straightforward reaction approach (Fig. 1.7C). Unfortunately, the resulting disulfide bond is relatively labile in biological fluids like serum. Another useful reaction scheme is based on the covalent linkage between aldehyde and hydrazide groups to form a hydrazide bond (Fig. 1.7D). Reactive aldehyde groups can be created by oxidation of carbohydrate groups. Obviously, mild oxidation conditions have to be used for the oxidation reaction on relatively unstable molecules like antibodies. Chemical reaction between two primary amines is another approach for bioconjugation applications (Fig. 1.7E). In this case, coupling can be achieved using homobifunctional crosslinkers such as glutaraldehyde [90, 91].

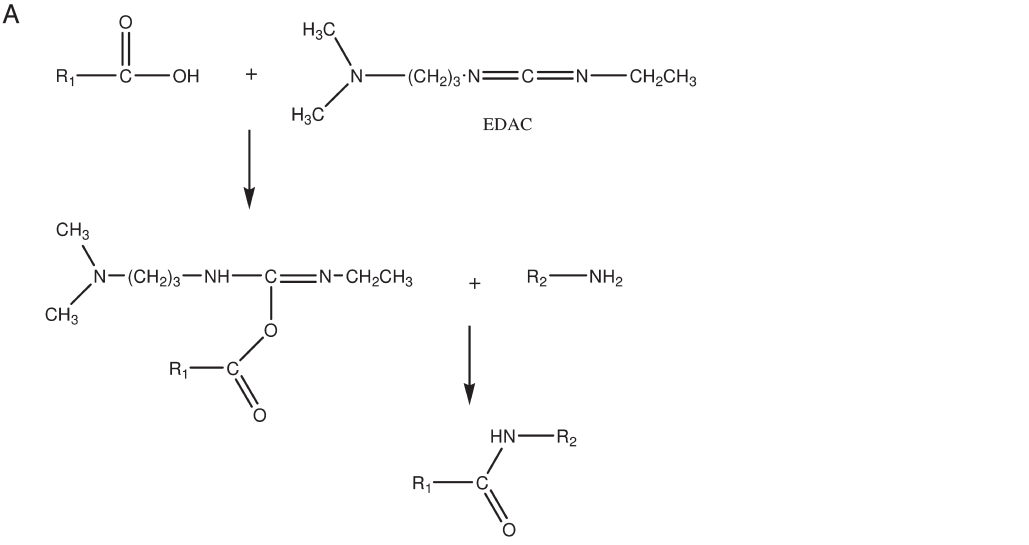
1.3.2 Bioconjugation of Polymeric Nanoparticles

From a bioconjugation perspective, polymeric nanoparticles are attractive because they can be prepared with various different reactive groups on their surface. The obvious benefit is that a comparably broad spectrum of conjugation chemistry approaches can be used, including covalent and noncovalent ones (Fig. 1.6). These different bioconjugation strategies are reviewed below.

1.3.2.1 Noncovalent Approaches

One of the most widely applied approaches in bioconjugating polymeric nanoparticles is simple physisorption of the biomolecules on the particle surface (Fig. 1.6A) [93–97]. Because biomolecule activity is compromised by uncontrolled adsorption, spacer molecules have been introduced (Fig. 1.6B). For example, nanoparticles first coated with Protein A (isolated from the cell wall of *Staphylococcus aureus*), which attaches specifically to the Fc fragment of the IgG antibody, have proper Fab orientation for antibody binding [97]. Unfortunately, such simple adsorption approaches are only of limited use when applied in serum. This is because serum proteins compete with antibodies for adsorption sites [93–95]. Furthermore, *in vivo* experiments have revealed that such simply designed nanoparticles tend to accumulate

A



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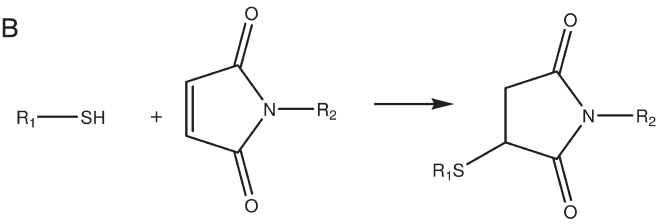

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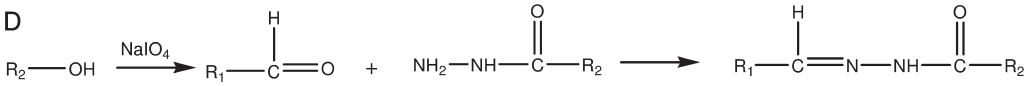

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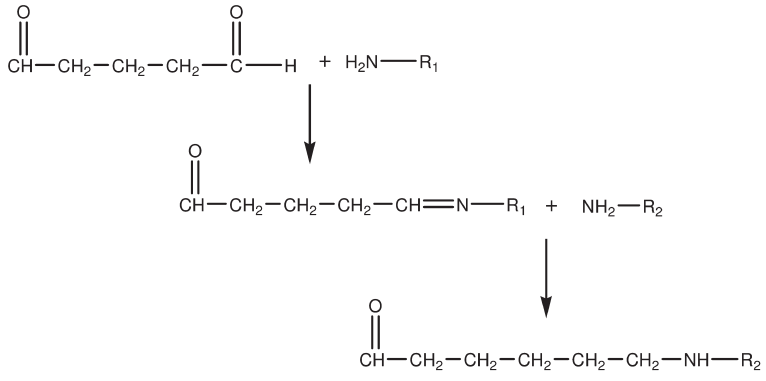

in the liver and spleen. Using a similar design strategy, polystyrene nanoparticles have been functionalized with humanized mAB HuEP5C7.g2 [98]. These delivery systems target cells expressing E and P-selectin.

1.3.2.2 Covalent Approaches

Obviously, the activity of ligands bound to nanoparticles strongly depends on the stability of the ligand–nanoparticle linkage and the proper orientation of the ligand. To address these needs, ligands have been bound covalently to polymeric nanoparticles using several different conjugation approaches (Fig. 1.6E) [7, 99–102]. For example, an amide linkage strategy was pursued to bind lactose to the poly(vinylamine)-grafted nanoparticles [99]. Such lactose-functionalized carrier systems are useful because they specifically bind to lectin RCA_{120}. The same amide-based linkage chemistry was used to bind proteins to dye encoded latex particles [7]. In another approach, transferrin was conjugated to PEG coated poly(cyanoacrylate) nanocrystals, with the transferrin bound to PEG via periodate oxidation [100]. A heterobifunctional linker was used to bind antibodies to BSA-containing nanoparticles [101, 102]. In this case, the crosslinker (glutaraldehyde) was bound to BSA to provide free aldehyde groups, which could then be linked to the primary amines of the antibodies. Another interesting strategy is based on formation of ligand-carrying nanoparticles via polymerization of monomers with bound ligands. Such a strategy has been applied to create nanoparticles with biotin groups, which then allowed for specific linkage to avidin [103, 104]. The grafting of carbohydrates to poly(L-lysine) was also reported [105]. This carrier can bind to carbohydrate-binding proteins on the surface of target cells.

1.3.3 Bioconjugation of Quantum Dots

Though quantum dots are very small, their surface area is large enough for linking to multiple biomolecules [106]. There are several ways to bind biomolecules to quantum dots. These involve either direct binding to the quantum dot surface or attachment via a stabilizing layer acting as a crosslinker between the ligand and reactive surface of the nanoparticle. In the first case, ligand binding can be achieved covalently or noncovalently. Direct covalent coupling is accomplished commonly by use of thiol coupling chemistry, although a silane-based coupling is needed if quantum dots carry a stabilizing silica shell. Noncovalent ligand-quantum dot coupling, however, is typically pursed using electrostatic or hydrophobic forces. On the basis of these general concepts, several bioconjugation strategies have been pursued. These are discussed below.

Fig. 1.7. Commonly used chemical reactions in the bioconjugation of fluorescent nanoparticles. Shown are the coupling chemistries between (A) carboxylic acid and primary amine, (B) thiol and maleimide groups, (C) two thiols to form a disulfide bond, (D) hydrazide and aldehyde groups, and (E) two primary amines. (Adapted from Nobs et al., 2004 [92].)

1.3.3.1 Noncovalent Approaches

An electrostatic coupling approach has been applied to link negatively charged CdTe quantum dots and positively charged enzymes [107]. Electrostatic coupling also was pursued in several applications to bind positively charged protein domains (pentahistadine segment) to oppositely charged alkyl-COOH-capped quantum dots [31, 42, 88, 108–112]. An alternative electrostatic-based coupling strategy has been used to bind nanocrystals coated with trimethoxysilylpropyl-urea and acetate groups with high affinity in the cell nucleus [44]. Here the binding of the silanized nanocrystal surface is controlled with an anionic silane reagent. Negatively charged quantum dots have also been electrostatically bound to positively charged Maltose Binding Protein [113]. Quite often, adsorbed proteins show a stabilizing effect on the quantum dot surface, thereby improving the optical properties of the probes. For example, a two-fold increase in fluorescence was observed after adsorption of 10–15 Maltose-Binding Protein Pentahistadine (MBP-5HIS) to the quantum dot surface [114].

1.3.3.2 Covalent Approaches

A relatively straightforward covalent bioconjugation approach is based on the replacement of thiol acids present on the quantum dot surface with thiolated biomolecules. This strategy has been used to attach oligonucleotides, DNA [41, 84] and bovine serum albumin (BSA) [86] to quantum dots. Proteins also have been conjugated to quantum dots by reacting carboxylic acid groups on the nanoparticle surface with amine groups of the proteins [115]. A similar strategy was used to covalently attach IgG and streptavidin to quantum dots. The IgG system was applied as a cancer marker, whereas the streptavidin system was utilized for labeling actin, microtubules and the cell nucleus [116].

In another application, antibodies have been bound to quantum dots via sulfo-NHS crosslinking [117]. In a different approach, reactive biotin was covalently linked to either surface sulfhydryl or amine functionalities, thus allowing for the biotinylation of the quantum dot surface and the subsequent binding to streptavidin [44]. A biotin–streptavidin coupling approach was chosen, for example, to bind quantum dots to fibroblasts [44]. In this case, the nanocrystals were functionalized with biotinamidocaproic acid 3-sulfo-*N*-hydroxysuccinimide ester and then allowed to bind to fibroblasts previously incubated with phalloidin–biotin and streptavidin. A combination of covalent and noncovalent coupling strategies was pursued by conjugating quantum dots stabilized with amphiphilic PEG lipids to DNA. Here, the DNA coupling was achieved by replacing $\frac{1}{2}$ of the PEG-PE phospholipids with amino PEG-PE. Thiol modified DNA was then covalently coupled to the amines on the surface of the quantum dot [118].

1.3.4 Bioconjugation of Metallic Nanoprobes

Most conjugation concepts described for the linkage of ligands to quantum dots can also be applied to metal nanoparticles, such as Au or Ag nanoprobes. Again,

surface modification is accomplished using thiol-coupling chemistry. The binding of biomolecules to these nanoparticles can be covalent or noncovalent. With respect to biofunctionalization, gold nanospheres are particularly attractive because gold shows excellent biocompatibility properties, and does not require additional surface chemical modification prior to bioconjugation. In addition, gold nanoparticles can be made with very narrow size distribution. This is advantageous if accurate control of the number of bound biomolecules per nanoparticle is required.

1.3.4.1 Noncovalent Approaches

One option for bioconjugation is the direct attachment of a biomolecule to the metallic nanoparticle surface. Short peptide chains such as tiopronin [119] and the tripeptide glutathione [120] have been conjugated directly to a gold surface. Globular proteins have also been directly conjugated. Miziani et al. conjugated globular proteins directly to the surface of silver sulfide nanoparticles [121]. In another application, gold nanoparticles were coated with BSA via electrostatic interactions under mild conditions [122]. Due to the bioinertness of gold, biomolecules directly bound on the gold surface can retain their activity, as shown, for example, for redox enzymes [123] and cytochrome c [124]. Biotin–streptavidin coupling concepts have also been used. For example, disulfide-modified biotin was conjugated to gold nanoparticles and subsequently reacted with streptavidin [125].

1.3.4.2 Covalent Approaches

Direct coupling of biomolecules to the surface of metal nanoparticles represents a very facile bioconjugation strategy. Among the different metals, this conjugation strategy is best suited for gold because of its excellent bioinertness. For example, thiol-modified DNA has been conjugated directly to gold particles [74, 126–128]. Direct thiol coupling was also pursued to conjugate SH modified PEG to gold-shelled silica nanoparticles [129]. Covalent bioconjugation to the nanoparticle can also be mediated by heterobifunctional crosslinker molecules. One example is the surface modification of iron oxide nanoparticles with a trifluoroethylester-terminal poly(ethylene glycol) (PEG) silane and the subsequent coupling of biomolecules via their terminal amine or carboxyl groups [130].

1.4 Design of Biocompatible Coatings

1.4.1 General Considerations

1.4.1.1 Overview

Though multiple bioconjugation strategies have been worked out, the routine application of fluorescent nanoparticles in biomedical imaging remains challenging. This is largely because not only appropriate conjugation between biomolecules to nanoparticles is required but other important criteria concerning biocompatibility

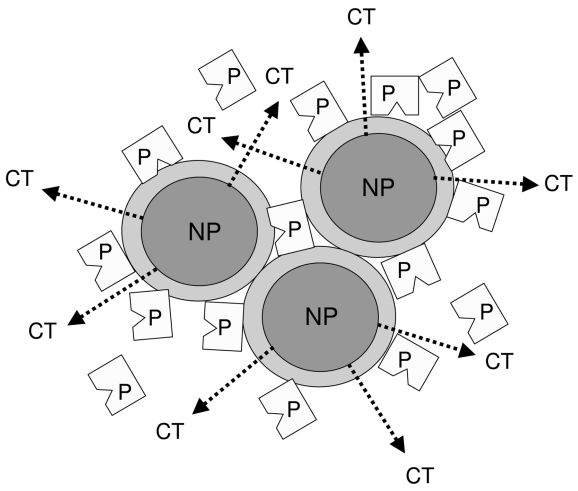

Fig. 1.8. Potential problems compromising the biocompatibility of fluorescent nanoparticles, including nanoparticle (NP) clustering, non-specific adsorption of proteins (P), and release of cytotoxic ions (CT).

have to be fulfilled. These criteria include (a) prevention of nanoparticle aggregation in a biological environment, (b) effective suppression of non-specific adsorption of biomolecules at the nanoparticle surface or their accumulation close to the surface, and (c) low cytotoxicity (Fig. 1.8). To address these criteria, nanoparticles need to be capped with protective coatings that show passivating surface properties. Notably, the first criterion, good colloidal stability, does not guarantee the biocompatibility of nanoparticles as, firstly, ions and proteins may be accumulated on or near the nanoparticle surface, thus varying the complex molecular composition nearby. Secondly, especially with heavy metal-containing quantum dots (e.g., CdSe, CdTe), individual nanoparticles may remain cytotoxic if the coating does not protect the biological environment from released heavy metals ions.

1.4.1.2 **Colloidal Stability**

The dispersion stability of nanoparticles is well-described by the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory, which predicts that such stability is determined by the balance between attractive van der Waals and repulsive electrostatic forces. As a result, nanoparticles are expected to remain stable (show no particle aggregation) if the strength of the repulsive electrostatic force exceeds that of the attractive van der Waals (vdW) force. In other words, nanoparticles show no aggregation if they contain a sufficient density of surface charges. Indeed, particles with charged surface properties do not aggregate in ion-free solutions. At the same time, this balance can clearly be shifted in favor of vdW forces if the charges on the particle surface are screened by counter ions in the solution. This is an important aspect to consider during chemical surface modification, because such

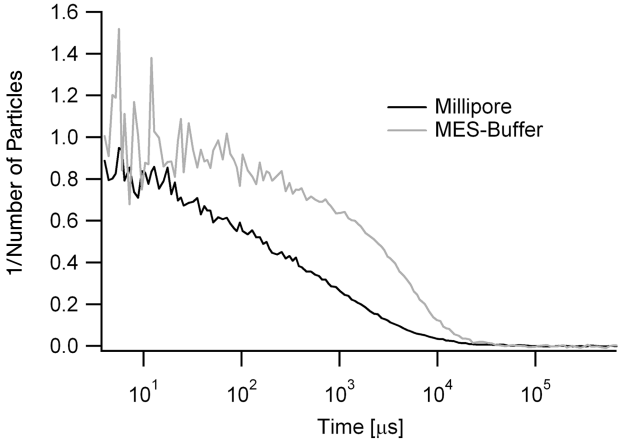

Fig. 1.9. Fluorescence correlation spectroscopy autocorrelation curves of ZnS-capped CdSe quantum dots surface-functionalized with carboxylic acid in Millipore water (dark curve) and 10 mM MES buffer (lighter curve). The notably higher diffusion time obtained for the buffer-based system indicates nanoparticle clustering.

modifications may result in surface charge neutralization, thereby compromising dispersion stability. More importantly, nanoparticles with charged surfaces tend to become unstable (show aggregation) in a biological environment. Figure 1.9 shows the loss of colloidal stability due to charge screening. Here comparative fluorescence correlation spectroscopy (FCS) autocorrelation curves from our laboratory are presented for CdSe/ZnS quantum dots with negatively charged carboxylic acid surface groups in Millipore water and in 10 mM MES buffer. As illustrated, the nanoparticles in MES buffer show notably higher diffusion times, indicating their increasing aggregation. If quantum dots are surface-functionalized with a layer of hydrophilic polymers such as poly(ethylene glycol) (PEG), colloidal stability can be achieved even in the presence of ions in solution. Such particle stabilization via polymer coatings is attributed to the flexibility of polymer chains and their repulsive "entropic spring" effect during particle–particle interaction. Currently, the concept of stabilization of nanoparticles due to the addition of polymeric coatings is widely accepted. Indeed, several experimental results seem to support the concept [131, 132]. Peptides adsorbed to the quantum dot surface can also have a similar stabilizing effect [133]. Conversely, it has been theoretically predicted that homopolymers, if end-grafted to nanoparticles in good solvents, may lead to nanoparticle attraction [134].

1.4.1.3 **Biocompatible Surfaces**

To make fluorescent nanoparticles 'bioinert', their surfaces need to be designed so as to prevent the non-specific adsorption of all relevant molecules in the biological medium. It is challenging to improve the design of surface coatings to a level of sophistication that fulfills this requirement. Fortunately, promising strategies for

the creation of bioinert surfaces are known from biomaterials research in the biomedical engineering community. As a result, our understanding about the host response of living tissue with respect to biomaterials has improved significantly and numerous sophisticated biomaterials have been developed [135]. A key aspect for the creation of colloidal solids with sufficient stealth properties in a cellular environment is the understanding of protein adsorption. Like surfactants, proteins have a high tendency to adsorb at interfaces. Interactions between proteins and surfaces are primarily noncovalent and include electrostatic, hydrophobic, and hydrogen-bonding interactions [136]. The different interactions result from the ''surface inhomogeneity'' of proteins, which typically are characterized by surface patches that may be charged, neutral, hydrophilic, or hydrophobic in character [137]. Consequently, surface properties of biomaterials affect the mechanism, rate, and extent of protein adsorption. Protein adsorption can be suppressed most efficiently on biomaterials whose surfaces are neutral, hydrophilic, and highly dynamic [138]. A paradigm of a protein-resistant surface is a solid substrate surface-functionalized with grafted PEG chains [139]. This is so because PEG is electrically neutral, thus minimizing electrostatic interactions, and highly hydrophilic, thus minimizing hydrophobic interactions. As the PEG chains are highly dynamic in an aqueous environment, the formation of strong hydrogen-bonding between protein and polymer is effectively suppressed. Besides those enthalpic effects, the high dynamics of the PEG chains results in a high entropy, which is also unfavorable for protein adsorption. It has been generally assumed that the surface resistance of PEG functionalized substrates is a result of the steric repulsion grafted PEG chains show for adsorbing proteins [138, 140, 141]. However, this assumption is valid only if the grafted PEG chains are of very high molecular weight and show large enough grafting densities to form polymer brushes [142]. Interestingly, Prime and Whitesides found that protein adsorption is prevented effectively even if the PEG chains have only two ethylene oxide segments, thus indicating that surface coverage is the most important parameter to prevent protein adsorption [143, 144]. In the same context, Single-chain Mean Field Theory (SCMF) calculations and experimental studies showed that protein adsorption on hydrophobic surfaces is prevented because PEG chains bind to the hydrophobic substrate, thereby blocking possible protein binding sites [145]. A later SCMF study by Szleifer and coworkers found that polymers with a low substrate affinity are more effective for kinetic control, whereas those with a higher affinity lead to a lower equilibrium concentration of adsorbed proteins [146]. Obviously, protein adsorption on substrates surface grafted with synthetic polymers is dependent on both the steric hindrance effect of the grafted polymer layer and the affinity of the underlying substrate for proteins and other molecules.

1.4.1.4 **Cytotoxicity**

Several potential processes lead to cytotoxicity. One source of nanoparticle-induced cytotoxicity, rather independent of particle composition, occurs if nanoparticles adsorb to cell surfaces [147, 148] or if they get ingested by cells [149, 150]. The cytotoxicity of CdSe/ZnS quantum dots is suggested to be due mostly to their inter-

action with cells [151]. Another source of nanoparticle-induced cytotoxicity occurs when the nanoparticle is composed of toxic materials that can be gradually released. A prominent example concerning fluorescent nanoparticles is the gradual release of heavy metal ions such as Cd^{2+} from CdSe or CdSe/ZnS quantum dots [152, 153]. Heavy metal ions are cytotoxic and often show several pathways of cytotoxicity. Indeed, Cd^{2+} may induce hepatotoxicity, immunotoxicity, and nephrotoxicity; apoptosis being a critical part of each toxicity type [154]. Studies concerning Cd-induced hepatotoxicity show, for example, the relevance of direct and indirect cytotoxic pathways [155]. The direct pathway is caused by Cd^{2+} binding to sulfhydryl groups on key mitochondrial molecules, thus damaging the mitochondria. The indirect pathway, though, is assumed to occur via activation of Kupffer cells. However, not all metals show such pronounced cytotoxicity. For example, gold nanoparticles have good biocompatibility properties [156]. From the above information, nanoparticles only show very low cytotoxicity if all potential sources of cytotoxicity are prevented efficiently.

1.4.2 Nanoparticle-stabilizing Coatings

To enhance the biocompatibility of nanoparticles, one popular strategy has been to cap the nanoparticles with polymeric coatings of high biocompatibility. In particular, poly(ethylene glycol) (PEG) has been applied as a coating material because of its excellent biocompatibility properties. Originally studied on flat macroscopic surfaces for biomaterials applications, PEG coatings have been widely applied to sub-micron sized delivery systems and imaging probes [48, 49, 116, 118, 131, 157, 158]. Delivery systems coated with PEG have notably longer circulation times in the blood, thus exceeding traditional non-nanoparticle-based delivery methods for the prolonged delivery of drugs, diagnostics, genes, and vaccines [159–162]. Based on the success of PEG coatings in drug delivery systems, similar coating strategies have been applied to the design of biocompatible coatings of nm-size imaging probes in biological and biomedical imaging. A facile approach for adding a PEG layer to the quantum dot surface is via physisorption of amphiphilic molecules [116, 118]. Here one interesting strategy has been to use PEG-containing lipopolymers, which result in excellent colloidal stability in *Xenopus* embryo cells over several days [118]. In an alternative amphiphilic passivation approach, block copolymers have been utilized to enable colloidal stability of quantum dots [116, 163, 164].

Chemically coupled PEG coatings have even greater stability. Wuelfing et al. reported the surface functionalization of gold nanoparticles using CH_3O-PEG-SH [131]. Covalent PEG coatings were also used to increase the colloidal stability of CdSe [49], CdS, [158] and SiO_2-capped CdS [48] quantum dots. However, in these cases, the PEG coatings lack reactive surface groups, thus preventing the further immobilization of ligands on the PEG layer. To overcome this limitation, nanoparticles need to be surface-functionalized via mixtures of mono-functional PEGs and other linker molecules [48] or via heterobifunctional PEG-linkers. Heterobifunc-

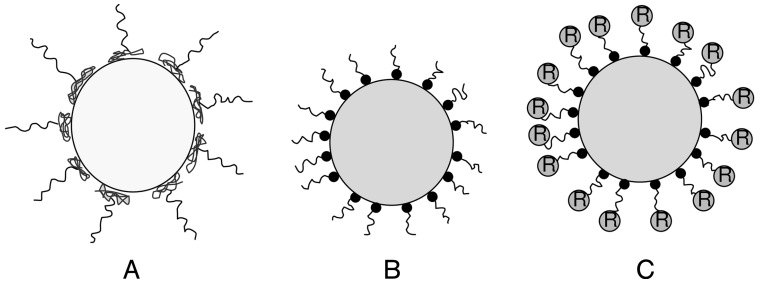

Fig. 1.10. Design of nanoparticles with stabilizing, biocompatible coatings, including (A) adsorption of amphiphilic molecules such as amphiphilic diblock copolymers; (B) chemical coupling of hydrophilic polymeric systems; and (C) linkage of spacer molecules with hydrophilic surface groups.

tional PEG-linkers can be synthesized via monoprotected symmetric PEG linkers, such as monoprotected diamines derived from tetraethylene glycol [165–167]. Another strategy for the synthesis of heterobifunctional PEG linkers is based on the ring-opening polymerization of ethylene oxide using a metal alkoxide initiator [168–171]. For example, α-acetal-PEG-SH has been synthesized using this strategy [172]. Here the facile modification of the acetal group into a reactive aldehyde allowed the immobilization of ligand molecules on the surface of PEGylated gold particles [173]. Meanwhile, various heterobifunctional PEG linkers are also commercially available. Figure 1.10 shows the design of a biocompatible surface coating where a few linkers support the targeted immobilization of ligand molecules. Such a design can be accomplished by using a mixture of homofunctional and heterobifunctional PEGs. An excess of homofunctional PEG molecules, which have no reactive surface groups after surface coating, suppresses the non-specific binding of proteins, whereas the heterobifunctional linkers facilitate the targeted coupling to ligands. Notably, however, a PEG layer does not prevent cytotoxic side reactions due to the leaching of toxic heavy metal ions from quantum dots. In those cases, an additional protective layer preventing the egress of harmful ions is essential [153].

PEG-based coatings are not the only biocompatible polymeric coatings. For example, poly(acrylic acid) has been used to stabilize luminescent silicon nanoparticles [174]. Because this anionic polyelectrolyte has a high density of carboxylic acid moieties, the immobilization of amine-containing molecules is straightforward. A similar concept has been applied to conjugate CdS quantum dots with aminodextran [175]. Dendrimers have also been used as stabilizing coating material [176]. Quantum dots have been embedded into larger polymeric spheres via a facile procedure using block copolymers [163, 177], and CdSe-ZnS quantum dots have been incorporated into glyconanospheres via electrostatic coupling with carboxymethyldextran and polylysine [178]. Several other procedures have afforded gold glyconanospheres [173, 179, 180]. Quantum dots capped with specific peptide coatings have recently been shown to be quite promising biocompatible imaging

probes that can be designed for targeted labeling of biomolecules [20, and references therein].

1.4.3
Low Cytotoxicity Coatings

As stated before, successful application of fluorescent nanoparticles in a biological environment requires not only high dispersion stability and the suppression of non-specific biomolecule adsorption, but also should show low cytotoxicity. This topic is particularly important for applications involving quantum dots where toxic heavy metal ions can be released [152]. If properly passivated, quantum dots seem to show no significant cytotoxicity. For example, a study on *Xenopus* embryos did not reveal significant cytotoxicity after injection of 2×10^9 and 5×10^9 CdSe/ZnS quantum dots per cell [118].

Several strategies have been pursued to suppress this oxidation process. One approach is simply to passivate the quantum dot surface with binding ligands [181]. Importantly, such a passivation not only lowers the cytotoxicity of nanoprobes, but also enhances their quantum yields. Quantum dots can be further passivated by adding a protecting semiconductor shell. Such coatings also significantly lower the cytotoxicity of CdSe quantum dots, but do not completely eliminate the problem [152]. Surface silanization is another promising approach to suppressing surface oxidation [44, 46, 182–184]. The stability of the coating is provided by crosslinks within the siloxane shell. Importantly, such shells are quite stable in a biological environment [46]. Furthermore, CdSe/ZnS quantum dots with a protective shell of crosslinked silica significantly reduce the release of Cd^{2+}, thus lowering the cytotoxicity of fluorescent nanoparticles [153]. Interestingly, the addition of a bovine serum albumin (BSA) layer further reduced the cytotoxicity of CdSe/ZnS quantum dots [152]. The BSA, added to the mercaptoacetic acid-functionalized quantum dot surface using EDC coupling, may act as a diffusion barrier for O_2 molecules.

Based on the above discussion, the appropriate biofunctionalization of nanoparticles clearly must address the features of colloidal stability, bioinertness, specificity with respect to target biomolecules, and low cytotoxicity. Figure 1.11 shows a biofunctionalization architecture where these crucial features are considered in its design.

1.5
Applications

Our growing ability to synthesize and surface-functionalize nanoparticles has provided new opportunities in bioanalytical and biological imaging applications. Metallic nanoparticles have been used primarily in bioanalytical projects. Nanoparticle-based imaging applications, however, rely more on dye-doped polymeric nanoparticles and quantum dots due to their excellent fluorescence proper-

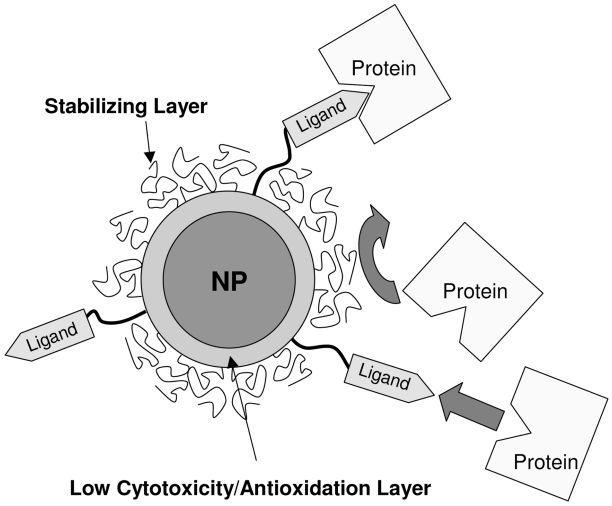

Fig. 1.11. Schematic of an appropriately biofunctionalized quantum dot. The inner coating protects from surface oxidation whereas the outer coating guarantees dispersion stability and biocompatibility. Immobilized ligands mediate the specific binding to biomolecules.

ties and photostability. While dye-doped systems have been used initially in such imaging applications, quantum dots have recently increasingly become the nanoparticle systems of choice. It is fair to say that further progress in bioanalytical and imaging applications is strongly linked to the availability of novel biofunctionalized nanoparticles of improved designs. Though developing rapidly, this highly interdisciplinary field is still in its infancy. The rapidly growing number of publications over the last decade reflect its relevance.

Though this chapter focuses primarily on the relevant *concepts* of biofunctionalization, this section describes recent *applications* using biofunctionalized fluorescent nanoparticles. We do not claim to review all published applications where such particles have been used because this is clearly beyond the scope of the chapter. Instead, the intent is to provide a representative overview of recent nanoparticle-related applications in the fields of diagnostics and live cell and *in vivo* imaging.

1.5.1 Biosensing

Fluorescent nanoparticles are highly promising probes for bioanalytical applications. In particular, their use in the field of biosensors is attractive because fluorescence-based techniques are extremely sensitive and nanoparticle probes show high photostability, thus allowing for long-term observations.

1.5.1.1 Polymeric Sensors

Bioanalytical applications based on polymeric nanoparticles are now well established. In one application, the combination of two silica precursors, tetraethylorthosilicate and phenyltriethoxysilane, was used to synthesize organic dye-doped silica nanoparticles. The nanoparticles were coated with avidin to determine biotinylated bovine serum albumin. In addition, a glutamate sensor was designed by immobilizing glutamate dehydrogenase on the nanoparticle surfaces [185]. In another application, a BODIPY-doped polymeric nanoparticle was designed for the detection of Cu^{2+}. Here the metal-chelating receptor, cyclam, was covalently bound to the surface of the nanoparticle. In the presence of Cu^{2+} the fluorescence emission of the BODIPY is quenched by the overlapping adsorption band of the metal–chelator complex [186]. Fluorescent polymeric nanoparticles have also been used for the intracellular monitoring of key biological components. Using three different nanoparticle fabrication technologies, sensors based on polyacrylamide, crosslinked decyl methacrylate, and silica-based sol–gel have been characterized in aqueous solution and intracellular surroundings. Each matrix can be combined with specific ''free dyes'', ionophores, or enzymes to produce sensors selective for the biological component of interest. These nano-optodes are termed PEBBLEs (probes encapsulated by biol. localized embedding). Spherical sensors less than 600 nm in diam. (and reducible to below 100 nm) have been made from all three matrices. Acrylamide-based sensors have been used to monitor intracellular pH and calcium (with proven selectivity over Mg^{2+}). Decyl methacrylate has been successfully applied to intracellular potassium monitoring with probes 1000× more selective for potassium than sodium. Such a sol–gel-based approach has also proven useful for monitoring intracellular oxygen at physiologically interesting concentrations. PEBBLEs, with a wide range of both simple and complex sensing schemes, provide a unique tool for minimally invasive intracellular monitoring, with many significant advantages over free dyes as well as over fiber-optic sensors [187–189].

1.5.1.2 Quantum Dot Sensors

The great potential of quantum dots for sensing applications has been demonstrated in several instances. In one application, quantum dots have been utilized as maltose sensors [114]. Here quantum dots were surface-functionalized with a maltose-binding protein (MBP) containing β-cyclodextrin-QSY-9 attached to the binding site to quench the quantum dot emission. The senor concept is that maltose displaces the β-cyclodextrin-QSY-9, thus inducing quantum dot emission. Another reported quantum dot-based maltose sensor using MBP acts as a double FRET sensor [114]. Here the quantum dot emission excites Cy3 that is adsorbed on the surface of the MBP. The Cy3 emission then excites the β-cyclodextrin–Cy3.5 whose emission is observed. Once maltose is introduced to the sensor, the Cy3 emission is no longer quenched by the Cy3.5, because the β-cyclodextrin–Cy3.5 has been displaced by maltose [114].

Quantum dots also show great promise in immunoassays. For example, an immunoassay has been performed by binding an antibody covalently to a glass chip,

followed by binding of the antigen. The antigen was then detected with the addition of antibody labeled quantum dots, which bound selectively to the captured antigen [190]. In another application, Tran et al. prepared quantum dot conjugates with the immunoglobin G (IgG) binding domain of streptococcal protein G (PG) appended with a basic leucine zipper attachment domain (PG-zb). They also demonstrated that the quantum dot/PG conjugates retain their ability to bind IgG antibodies, and that a specific antibody coupled to quantum dots via the PG functional domain efficiently binds its antigen. Preliminary results indicated that electrostatically self-assembled quantum dot/PG-zb/IgG bioconjugates can be used in fluoroimmunoassays [191]. Quantum dot–antibody conjugates were again successfully used in fluoro-immunoassays to detect both a protein toxin (staphylococcal enterotoxin B) and a small molecule (2,4,6-trinitrotoluene) [108]. Goldman et al. described a conjugation strategy employing an engineered molecular adaptor protein, attached to the quantum dots via electrostatic/hydrophobic self-assembly, to link quantum dots with antibodies. Quantum dots can also be used as sensors for sugars; a quantum dot FRET application using adsorbed MBP has been mentioned [114].

Powerful sensor concepts can also be designed using the multiplexing capability of quantum dots. This results from the broad excitation and narrow, size-dependent emission bands of quantum dots. This concept has been used, for example, to embed quantum dots of six different colors and ten intensities per color in nanoparticles composed of styrene, divinylbenzene, and acrylic acid. This combination can, theoretically, code one million nucleic acids or protein sequences [80]. In another application, a high-throughput assay has been developed for the parallel detection of antibodies using quantum dot microbeads. In this case, a custom designed microfluidic chip with multiple micro-wells was utilized for capturing of microbeads, antibody injection into each micro-well, QD injection, and fluorescence detection [192]. These beads could be identified with a standard flow cytometer at a rate of 1000 beads s^{-1} [193].

1.5.1.3 Metallic Sensors

Gold nanoparticles (2.5 nm) have been used to recognize and detect specific DNA sequences and single-base mutations. Detection is based on fluorescently tagged, thiol modified oligonucleotides bound to the surface of the gold nanoparticle. Upon self-assembly, the fluorophores arch toward the gold surface, quenching the fluorescence. Upon binding the appropriate target sequence, the oligonucleotide undergoes a conformational change, moving it away from the gold nanoparticle surface, and restoring the fluorescence [194]. Another interesting approach to ultrasensitive detection of DNA hybridization is based on Au nanoparticle-amplified surface plasmon resonance (SPR). Interestingly, even without further optimization, the sensitivity of this technique begins to approach that of traditional fluorescence-based methods for DNA hybridization and oligonucleotide detection [62]. Gold nanoparticles have also been used in the detection of glucose and glucose oxidase. The growth of the Au nanoparticles requires H_2O_2 and converts it into O_2. In the presence of glucose oxidase and glucose, O_2 is converted into

H_2O_2, allowing the Au nanoparticles to grow, thus giving a shift in absorbance over time [195]. Finally, Ag has also been employed for surface plasmon-based nanosensors concerning the detection of Alzheimer's disease. Autopsied brain samples of humans with Alzheimer's disease show elevated levels of amyloid-derived diffusible ligands (ADDLs), which suggest that a definitive chemical diagnosis of Alzheimer's could be achieved with a sensitive method to detect ADDL or anti-ADDL antibodies. The λ_{max} of silver nanoparticles conjugated with anti-ADDL was monitored before and after the incubation with ADDL. The resulting $\Delta\lambda_{max}$ indicated that it is possible to monitor the interaction between ADDL and anti-ADDL using Ag nanoparticles. While undergoing a promising initial trial, the practicality of the system as a sensor is limited because its specificity becomes low at nM concentrations of anti-ADDLs [196].

1.5.2 Fluorescent Nanoparticles as Labels in Biological Imaging

1.5.2.1 Dye-doped Nanoparticles

Though very small, fluorescent dyes are only of limited applicability in biological imaging because of their poor photostability and limited brightness. To improve the photostability, initially, dye-doped nanoparticles have been used as photostable imaging probes. For example, 20 nm dye-doped latex nanoparticles have been linked to DNA-binding proteins (restriction enzyme EcoR1) to probe specific sequences on stretched DNA [7]. In addition, ruthenium bipyridyl-doped silica nanoparticles can be used to stain and image leukemia cells [4]. More recently, Ru(bpy)-doped silica nanoparticles have been applied successfully in several fluorescent imaging applications, including immunocytochemistry, immunohistochemistry, and DNA and protein microarrays [197]. Here the nanoparticles were surface-functionalized with avidin, thus allowing straightforward coupling to biotinylated antibodies. In one case, avidin-capped, dye-doped silica nanoparticles were conjugated with biotinylated mouse anti-hIgM to stain human peripheral blood mononuclear cells. In another case, these photostable nanoprobes were coupled to biotinylated goat anti-ChAT antibodies to label choline acetyltransferase to image mouse brain tissue. Interestingly, these dye-doped silica nanoparticles are not outperformed by quantum dots with respect to photostability.

1.5.2.2 Quantum Dots

Among the different types of fluorescent nanoparticles, quantum dots seem to show the greatest promise as labels in biological imaging applications. Since their introduction as labels in cellular imaging in 1998 [44, 115], multiple reports have shown the strengths of these nanoprobes for tagging and imaging applications in biological systems. This strength is based on the impressive photostability of quantum dots and on their broad excitation and narrow size-dependent emission properties. Most importantly, the high photostability guarantees the observation of biomolecules over longer time periods. Dubertret et al. have reported a very elegant example of such a long-term observation of biological processes [118]. Here, quan-

tum dots with biocompatible coatings were injected into *Xenopus* embryos and their movement into different cells during tadpole development was monitored over several days.

Quantum dot multi-color imaging in cells is another fascinating imaging approach. For example, quantum dots biofunctionalized with streptavidin and IgG have been used to label the breast cancer marker Her2 on fixed and live cancer cells, to tag actin and microtubules, and to stain nuclear antigens inside the nucleus [116]. Quantum dots have also been shown to be useful in studying ATP driven biological processes. The *in vitro* sliding of quantum dot labeled actin filaments was observed over periods of 10–12 s [198]. The chaperonin proteins GroEL (from *Escherichia coli*) and T.th ('T.th cpn', from *Thermus thermophilus* HB8) typically encapsulate denatured proteins within a cavity and release them in the presence of ATP. This encapsulation and release technique was used to give quantum dots high thermal and chemical stability in various aqueous mediums, while also allowing the controlled release of quantum dots [199].

Quantum dot labels are particularly advantageous over traditional organic dyes in single-molecule tracking applications because dye-based tracking experiments are limited by very short observation times [200, 201]. Quantum dot-based single molecule tracking in biological systems represents a fascinating emerging research area. The first results indicate great promise. Dahan et al. have successfully tracked individual quantum dot-tagged glycine receptors in the neuronal membrane of live cells and analyzed the diffusion properties [202]. Furthermore, this technique allowed them to observe the entry of individual glycine receptors into the synapse, which was confirmed by electron microscopy experiments. The ability to detect quantum dots not only optically but also by electron microscopy is clearly a very powerful feature. Another recent single-molecule application was related to the monitoring of quantum dot-labeled epidermal growth factor. In this case, a new transport process was discovered [203].

Fluorescent nanoparticles have also been applied in animal imaging experiments; again, quantum dots seem to show great promise. Proper biocompatible surface coating is essential for long-term stability of the probes in a biological environment [204]. In another application, quantum dots have been targeted *in vivo* via peptides immobilized on the quantum dot surface [205]. Here, several types of peptides were used to label different regions of the mice tissue. Thus, quantum dots with lung-targeting peptides were shown to accumulate in the lungs of mice whereas other peptides targeted quantum dots to blood or lymphatic vessels in tumors. Targeted quantum dot transport in live animals was recently reported in mice [164]. In this case, PEG-coated nanoprobes were used that carried conjugated antibodies directed against prostate-specific membrane antigens. A clear boost in animal imaging should be expected from the recent development of quantum dots with emission bands in the near-infrared (NIR) because this wavelength range offers an optical window for tissue imaging. Indeed, NIR quantum dots have been applied successfully to conduct imaging experiments on rat and porcine tissue [206, 207]. In one case, rat coronary vasculature was studied. In the other case, porcine sentinel lymph nodes were imaged. Interestingly, when injected interdermally

on the thigh of a 35 kg pig, doctors could visualize the nearby sentinel lymph nodes within 3–4 min and use the infrared signal as a guide during surgery. Despite the relatively low power NIR excitation used (5 mW cm^{-2}) the sentinel lymph nodes were observed as deep as 1 cm below the skin surface.

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