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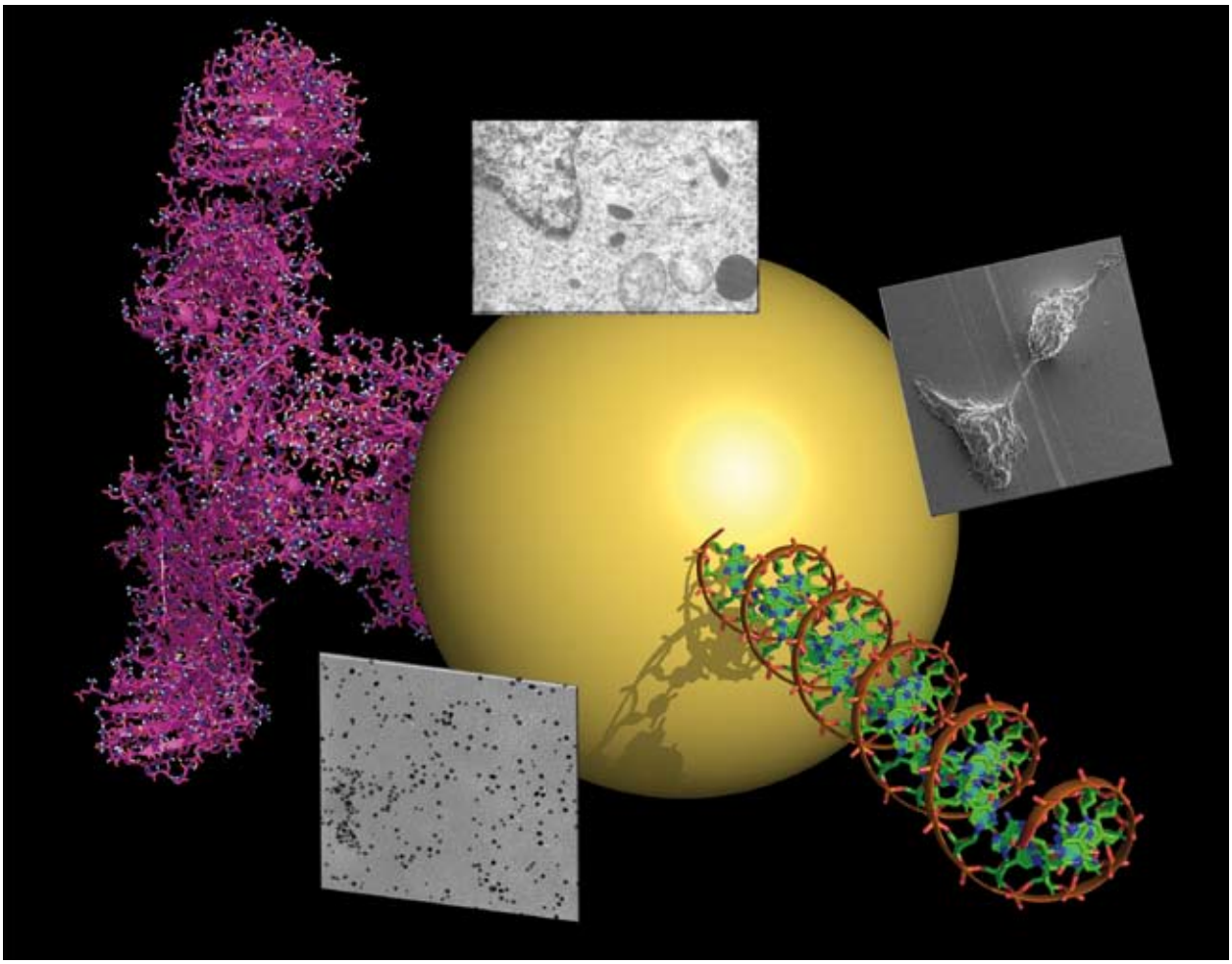

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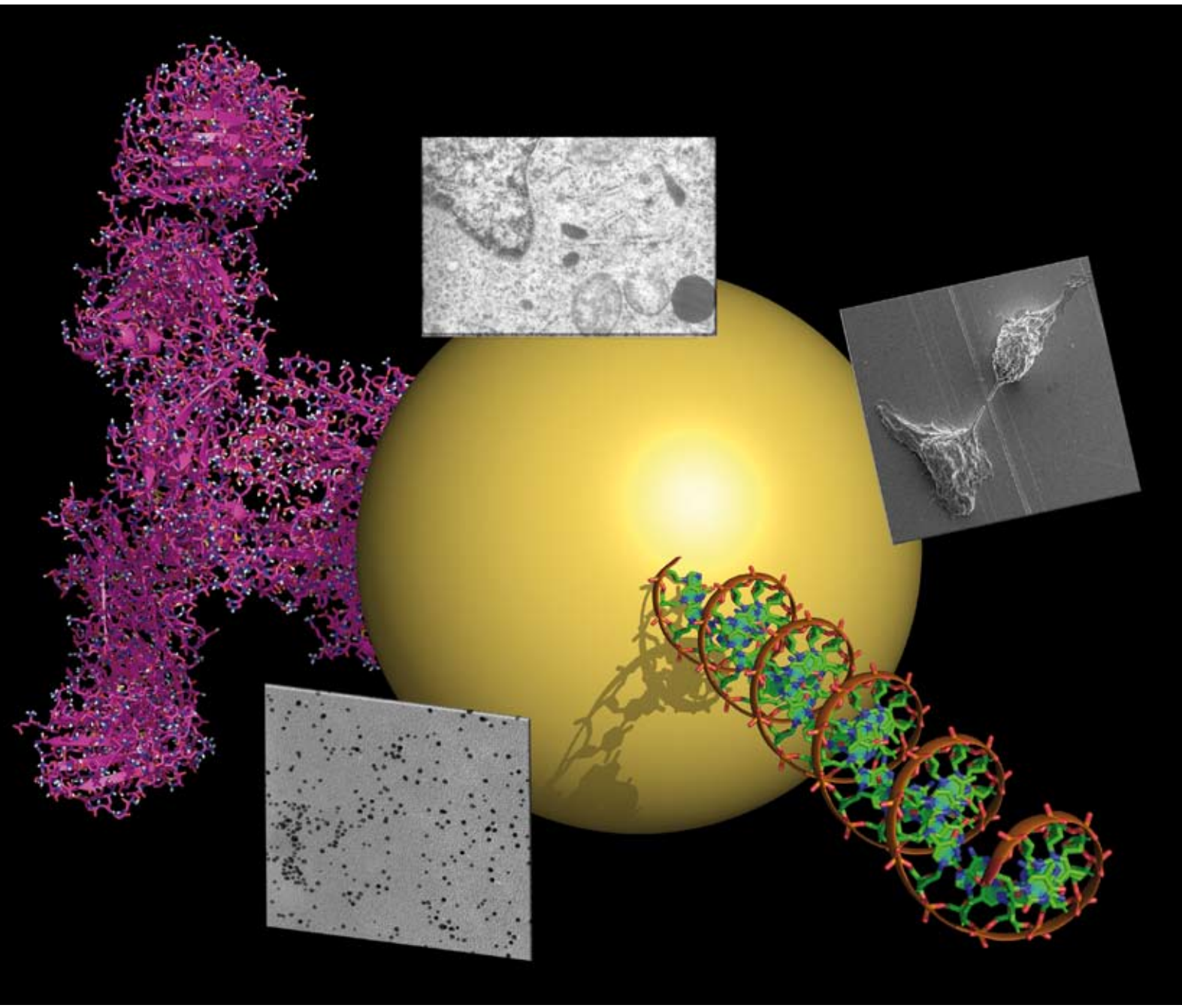

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Biological applications of gold nanoparticles†

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This *critical review* gives a short overview of the widespread use of gold nanoparticles in biology. We have identified four classes of applications in which gold nanoparticles have been used so far: labelling, delivering, heating, and sensing. For each of these applications the underlying mechanisms and concepts, the specific features of the gold nanoparticles needed for this application, as well as several examples are described (142 references).

1. Introduction

Colloidal gold nanoparticles have been used technologically since ancient times due to their optical properties, in particular for staining glass. Systematic investigations on gold colloids go back to the days of Faraday, though in their use for biological applications the breakthrough happened only in the last decade.[1] This goes hand in hand with the advent of (bio-) nanotechnology, which nowadays allows for controlled synthesis and functionalization of materials on the nanometre scale and thus provides a toolbox that did not exist before. It is legitimate to ask whether the increasing use of gold nanoparticles in biology is just an effect of the ''nano-hype'', or whether colloidal gold offers particular properties which go beyond the performance of previously used materials or even allow for unprecedented techniques. The purpose of this review is to outline the conceptual properties of colloidal gold nanoparticles and to outline the motivation for their use in different areas of biologically related research. We have classified the uses of gold nanoparticles into four concepts of applications: labelling, delivering, heating, and sensing.

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2. Some aspects of the synthesis and properties of gold nanoparticles

2.1 Synthesis and phase transfer

The synthesis of gold nanoparticles with diameters ranging from a few to several hundreds of nanometres is well established in aqueous solution as well as in organic solvents. In typical syntheses, gold salts such as $AuCl_3$ are reduced by the addition of a reducing agent which leads to the nucleation of Au ions to nanoparticles. In addition, a stabilizing agent is also required which is either adsorbed or chemically bound to the surface of the Au nanoparticles. This stabilizing agent (often also called a surfactant) is typically charged, so that the equally charged nanoparticles repel each other so that they are colloidally stable. For the most common synthesis route in

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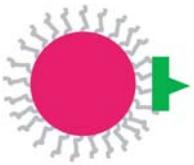

Fig. 1 Schematic of a ligand-conjugated gold nanoparticle. The gold core (red) is surrounded by stabilizer molecules (grey) which provide colloidal stability. Ligands (green) can be either linked to the shell of stabilizer molecules (as shown here) or directly attached to the gold surface by replacing part of the stabilizer molecules.

aqueous solution, citric acid serves first to reduce the gold salt and thus to trigger nucleation, and secondly by adsorption to the particles it provides colloidal stability to the particles by its negative charges.[2,3] Similar synthesis routes can also be performed in organic solvents,[4,5] though in this case the reducing agent is different from the stabilizing agent. For particles dispersed in organic solvent frequently surfactants based on hydrophobic alkane chains are bound to the particle surface in order to provide colloidal stability. A sketch of the geometry of Au nanoparticles is given in Fig. 1. Besides growing of gold particles of spherical shape other geometries such as rod-shaped particles or hollow shells can also be synthesized.[6,7]

2.2 Surface modification and bioconjugation

Colloidal gold nanoparticles are surrounded by a shell of stabilizing molecules. With one of their ends these molecules are either adsorbed or chemically linked to the gold surface, while the other end points towards the solution and provides colloidal stability. After synthesis of the particles the stabilizer molecules can be replaced by other stabilizer molecules in a ligand exchange reaction. As thiol moieties bind with high affinity to gold surfaces, most frequently thiol-modified ligands are used which bind to the surface of the Au particles (which are by several groups also called ''monolayer-protected clusters'') by formation of Au–sulfur bonds.[8] Ligand exchange is motivated by several aspects. Ligand exchange allows, for example, the transfer of Au particles from an aqueous to an organic phase (and *vice versa*) by exchanging hydrophilic surfactants with hydrophobic surfactants (and *vice versa*).[9] In this way, by choosing the surfactant molecules, it is possible to adjust the surface properties of the particles.

For applications in aqueous solution typically thiol-based surfactants with carboxylic groups at the other end pointing towards the solution are used. These molecules provide colloidal stability due to their negative charges; in addition they can also be used as anchor points for the further attachment of biological molecules. Often poly(ethylene glycol) (PEG) is used as a ligand as PEG reduces nonspecific adsorption of molecules to the particle surface and it provides colloidal stability because particles with PEG brushes on their surface repel each other for steric reasons.[10] Other surface coating techniques such as embedding particles in a silica shell have been used by several groups.[11]

Biological molecules can be attached to the particles in several ways. If the biological molecules have a functional group which can bind to the gold surface (like thiols or specific peptide sequences), the biological molecules can replace some of the original stabilizer molecules when they are added directly to the particle solution. In this way molecules like oligonucleotides, peptides or PEG can be readily linked to Au particles and subsequent sorting techniques even allow particles with an exactly defined number of attached molecules per particle to be obtained.[12,13] Alternatively, biological molecules can also be attached to the shell of stabilizer molecules around the Au particles by bioconjugate chemistry. The most common protocol is the linkage of amino-groups on the biological molecules with carboxy groups at the free ends of stabilizer molecules by using EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-HCl).[14,15] With related strategies almost all kinds of biological molecules can be attached to the particle surface. Though such protocols are relatively well established, bioconjugation of Au nanoparticles still is not trivial and characterization of synthesized conjugates is necessary, in particular to rule out aggregation effects or unspecific binding during the conjugation reaction. In particular, in many conjugation protocols the number of attached molecules per gold nanoparticle is only a rather rough estimate, as no standard method for determining the surface coverage of particles modified with molecules has yet been established.[16,17]

2.3 Cytotoxicity

Although gold nanoparticles are composed of an inert material, biocompatibility issues have to be considered. Cells exposed to gold nanoparticles will incorporate the particles (similar to nanoparticles of other materials) and the particles are stored inside the cells in perinuclear compartments, vesicular structures close to the cell nucleus.[18,19] Due to particle internalization cells or tissues in contact with gold nanoparticles will be exposed to the particles for extended periods of time. Concerning cytotoxic effects[20] one has to distinguish between effects related to the nature of the material (here: gold) and effects common to nanoparticles of even inert materials. Also for inert particles such as gold, inflammatory effects in tissues caused by particles have been demonstrated. However, in cell culture experiments Au nanoparticles are regarded as biocompatible, and acute cytotoxicity has not been observed so far.[21] In particular, no release of toxic ions as in the case of cadmium-based nanoparticles[22] has been reported. On the other hand, there are few examples of toxic effects related to the nature of Au, which might depend on the cell line,[19,23] on surface chemistry,[24] and on the nanoparticle size.[25] Actin fibres inside the cell, for example, can be affected by the presence of nanoparticles,[26] and very small Au-clusters have been demonstrated to fit into the grooves of DNA molecules and thus cause cytotoxic effects.[25,27] A more detailed discussion can be found in another article in this issue.[28]

3. Gold nanoparticles for labelling and visualizing

Traditionally, Au nanoparticles have been primarily used for labelling applications. In this regard, the particles are directed and enriched at the region of interest and they provide contrast for the observation and visualization of this region. The particles are used here as ''passive'' reporters; there is no change of particle properties required for the read-out as is the case for active sensor applications (see section 6). Gold nanoparticles are

a very attractive contrast agent as they can be visualized with a large variety of different techniques. The most prominent detection techniques are based on the interaction between gold nanoparticles and light.[29] Gold particles strongly absorb and scatter visible light. Upon light absorption the light energy excites the free electrons in the Au particles to a collective oscillation, the so-called surface plasmon.[30] In particular, close to the plasmon resonance frequency the absorption cross-section is very high. The excited electron gas relaxes thermally by transferring the energy to the gold lattice; finally the light absorption leads to heating of the gold particles.

Interaction with light can be used for the visualization of particles in several ways. Gold particles larger than around 20 nm can be directly imaged with optical microscopy in phase contrast or differential interference contrast (DIC) mode.[31] In dark field microscopy only light scattered from gold particles is detected with an optical microscope[32] whereby particles larger than 20–30 nm can be imaged. As the colour of the light scattered by gold particles depends on their sizes and shapes, gold particles can be used for labelling with different colours.[30,33] For small particles the scattering cross-section decreases rapidly whereas the absorption cross-section decreases less.

Absorbed light ultimately leads to heating of the particles and upon heat transport subsequently to heating of the particle environment. This can be observed in two ways. Photothermal imaging records density fluctuations (*i.e.* local variations of the refractive index) of the liquid environment around the particles by DIC microscopy.[34,35] Photoacoustic imaging, on the other hand, makes use of the fact that the liquids expand due to heat. A local heat-pulse due to light absorption leads to expansion of the liquid surrounding the gold particles and thus to the creation of a sound wave which can be detected by a microphone.[36,37] Both photothermal and photoacoustic imaging make use of the large light absorption cross-section of gold nanoparticles. Small gold particles have recently also been reported to emit fluorescence upon photo-excitation and thus can be visualized with fluorescence microscopy.[38,39] All of the above mentioned methods involving photoexcitation (phase contrast/interference contrast microscopy, dark field microscopy, photothermal imaging, photoacoustic imaging, and fluorescence microscopy) provide sufficient sensitivity to allow for detection at the single particle level.

Besides the interaction with visible light, the interaction with both electron waves and X-rays can also be used for visualization of Au nanoparticles. Due to their high atomic weight Au nanoparticles provide high contrast in transmission electron microscopy (TEM).[40] Au particles also scatter X-rays efficiently and thus provide contrast in X-ray imaging,[41] see also section 3.3. Finally, Au nanoparticles can also be radioactively labelled by neutron activation[42] and can be detected in this way by gamma radiation.

3.1 Immunostaining

Immunostaining is one of the traditional uses of Au nanoparticles in biology before the advent of "nanobiotechnology". The idea of immunostaining is the labelling of specific molecules or compartments of cells (see Fig. 2a) by antibodies. Without labelling, the molecules or cell compartments of interest cannot be visualized because of a lack of contrast with the other structures or molecules of the cell. For immunostaining, cells are typically fixed and permeabilized and Au nanoparticles are added that are conjugated with antibodies specific against the molecules of interest. Guided by molecular recognition, the antibody-modified Au particles will bind to the molecule (antigen) or target regions containing the antigen. As the cells are fixed and permeabilized, targets outside as well as inside cells can be labelled with gold particles in this way. The Au particles then provide excellent contrast for TEM imaging with high lateral resolution[43] and larger structures can also be imaged with optical microscopy.[44] Compared to fluorescence labelling, Au particles are more stable as they do not suffer from photobleaching which is a major limitation for fluorescence based methods, and in the case of TEM imaging better lateral resolution with high contrast can also be obtained. In immunostaining, molecules/structures are labelled with an excess of Au nanoparticles so that virtually all entities are labelled (possibly with several markers) in order to provide high contrast. In this case the local density of Au particles at the sites where the labelled molecules/structures are present is quite high. Therefore the resolution limit of the optical microscope (of a few hundreds of nm) does not allow for optically resolving individual molecules labelled by antibody-conjugated Au nanoparticles (of a few tens of nm in diameter), as the distance between adjacent Au nanoparticles is smaller than the optical resolution limit. However, due to better lateral resolution in electron microscopy, individual receptors can be resolved by visualizing the bound Au nanoparticles with TEM.

Immunostaining is also possible without fixing and permeabilizing cells, but in this case only structures/domains on the surface of the cell can be labelled. For immunostaining of the outer cell surface photoacoustic imaging can also be used besides the imaging techniques mentioned above. Photoacoustic imaging provides an additional feature in contrasting. When Au nanoparticles come close together (*i.e.* form small aggregates) the frequency of the plasmon resonance shifts to higher wavelengths. When freely dispersed colloidal Au nanoparticles are optically illuminated at wavelengths well above their plasmon resonance, the light is not absorbed and thus there will not be any photoacoustic signal. Small aggregates of Au nanoparticles, on the other hand, can absorb light at wavelengths above the plasmon resonance of freely dispersed, single Au particles. If, therefore, light with a wavelength above the plasmon resonance of freely dispersed Au particles is used for excitation there will be a photoacoustic signal for aggregates of Au nanoparticles, but not for single dispersed Au nanoparticles. If Au nanoparticles modified with antibodies against membrane receptors are used they will bind to the regions of the outer cell surface where the receptors are present. As several Au nanoparticles will bind to such regions there is a local "aggregation" of Au nanoparticles present which can provide a photoacoustic signal, whereas Au nanoparticles which are still in solution or which are randomly distributed on the cell surface due to nonspecific adsorption will not exhibit any photoacoustic signal.[36]

3.2 Single particle tracking

The molecules/structures on the outer cell surface can be labelled with Au nanoparticles which are conjugated with specific antibodies against these molecules/structures. In

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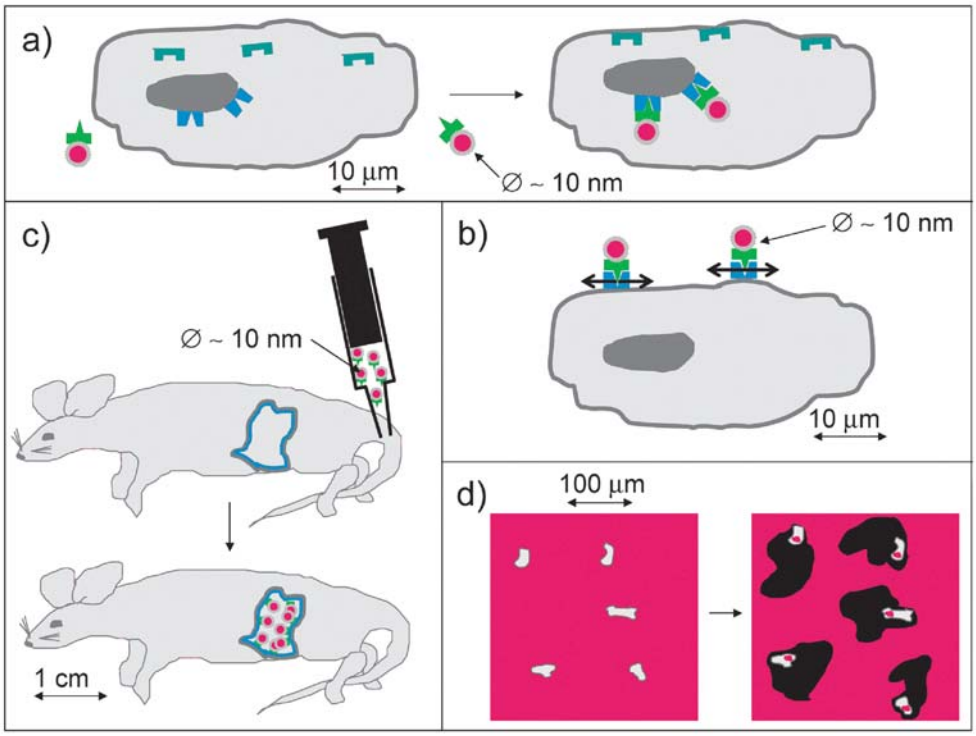

Fig. 2 Labelling with gold nanoparticles. Gold nanoparticles (core in red, stabilizing shell in grey) are conjugated with ligands (green) which bind to specific receptors (blue) but not to other structures (as the receptors shown in dark green). (a) Immunostaining: Gold nanoparticles conjugated with ligands against the structures to be labelled are added to fixed and permeabilized cells (shown in grey). Guided by molecular recognition they bind to the designated structures which are in this way stained with gold particles. In the image the particles are conjugated with ligands that bind to receptors on the surface of the nucleus, but not to other receptors, for example present at the inner cell membrane. (b) Single-particle tracking: Gold particles conjugated with ligands specific for membrane-bound molecules are added to living cells. In this way individual membrane-bound molecules are labelled with gold particles and their diffusion within the cell membrane can be traced *via* observation of the gold particles. (c) X-ray contrasting: Gold particles conjugated with ligands that permit specific uptake in target organs are injected into the bloodstream of animals. The organ can then be visualized by X-ray tomography due to the locally enriched gold particles. (d) Phagokinetic tracks: a surface is covered with a layer of gold nanoparticles. When cells (shown in grey) are cultured on top of the surface they will ingest the underlying nanoparticles. Upon cellular migration along the surface the cells incorporate all nanoparticles along their pathway leaving behind an area free of nanoparticles, which is a blueprint of their migration pathway. Images are not drawn to scale and important length scales are indicated in the images.

contrast to immunostaining, for single particle tracking only a few antibody-conjugated Au nanoparticles are added, so that after binding to the cell the particle labels present on the cell surface are very diluted. As in this case the average distance between adjacent Au nanoparticles is larger than the optical resolution limit, individual Au nanoparticles can be optically resolved. For imaging the movement of molecules/structures, living cells without fixation and permeabilization have to be used, which limits the labelling of molecules/structures to the outer cell surface and the visualization to optical techniques (phase/interference contrast microscopy, dark field microscopy, photothermal imaging, fluorescence imaging, or in combination with acoustics: photoacoustic imaging).

Most frequently, membrane-bound receptor molecules are investigated by single particle tracking. By time-resolved imaging of the receptors which are labelled with Au nanoparticles, their trajectories and thus their diffusion within the cell membrane can be observed (see Fig. 2b).[45] Movement of Au nanoparticles larger than around 40 nm can be traced directly with phase contrast or differential interference contrast microscopy.[46] For gold particles larger than around 20–30 nm the light scattered by individual Au nanoparticles can be recorded with dark field microscopy.[47] Movement of receptors labelled with even smaller Au nanoparticles (down to 5 nm) has been visualized with photothermal imaging.[48,49] Also other labels are used for the tracking of single receptors on cell surfaces and each of the methods has certain intrinsic advantages and disadvantages. The larger the particle label is, the easier (with regard to the required set-up) it can be optically imaged. The principle is the same for 40 nm Au nanoparticles as for latex and silica beads, whereby the beads can be impregnated with organic fluorophores and thus can be imaged with fluorescence microscopy.[50]

One could argue that gold nanoparticles can be more easily conjugated with antibodies than latex/silica beads (due to the thiol–gold chemistry), though surely the fluorescence of beads provides a better signal-to-noise ratio. At any rate, attachment of particles larger than around 40 nm to receptors might severely change the diffusion properties of the receptor molecules. For this reason the real challenge of single particle tracking is to use an as small (and as stable) label as possible. Organic fluorophores exist that are smaller than colloidal particles; however, they suffer from photobleaching and therefore their fluorescence can be traced only for limited periods of

time. Alternatively, colloidal fluorescent semiconductor nanoparticles (so-called quantum dots) can be used for single particle tracking of membrane molecules.[51] Though they are slightly larger than small organic fluorophores they suffer much less from photobleaching and thus allow for extended observation periods. Colloidally stable quantum dots are typically larger than 10 nm in diameter, as sophisticated coatings are needed to ensure colloidal stability. On the other hand, movement of gold particles down to 5 nm diameter can be imaged with photothermal microscopy[48,49] and as no fluorescence detection method is used, there is no limitation in observation time by photobleaching. For this reason photothermal microscopy of small Au nanoparticles is particularly advantageous when long periods of observation are required.

3.3 Contrast agents for X-rays

Whereas immunostaining and single particle tracking are used for visualizing structures within single cells, the same concept can also be applied for providing contrast *in vivo* to whole organs in animals and potentially in humans. Again the Au particles are conjugated with antibodies or ligands which bind as specifically as possible to the organ of interest in the animal. When particles are administered to the blood circulation a part of them will eventually bind *via* receptor–ligand interaction at the designated organ. The particles bound to the organ provide contrast for imaging and resolving the structure of the organ (see Fig. 2c). However, the big general problem of contrasting organs with colloidal nanoparticles is their short circulation time in the bloodstream, so that only a fraction of the particles has a chance to bind to the designated organ whereas a significant part is cleared from the bloodstream by the liver and kidneys. On the other hand, colloidal nanoparticles can provide better contrast compared to organic molecules. Gold nanoparticles can, for example, be imaged with high signal-to-noise ratio with X-ray computer tomography[41,52] and therefore only short exposure times are required, which helps to reduce radiation damage to surrounding tissues. X-rays penetrate skin and therefore organs deep inside the body can be imaged or addressed for therapy.[53] Furthermore, X-ray tomography set-ups are readily available in many hospitals. Here again, Au nanoparticles have to compete with fluorescent semiconductor nanoparticles (quantum dots).[54,55] In order to reduce the X-ray exposure of patients certainly fluorescence detection would be preferable compared to imaging with X-rays. On the other hand, light is absorbed by tissues even partly in the infrared (IR), so that fluorescence contrasting of organs deep inside the body is complicated. Furthermore colloidal gold nanoparticles are likely to cause less cytotoxic damage than the generation of colloidal quantum dots presently used.

3.4 Phagokinetic tracks

Albrecht-Bühler has introduced an innovative way of imaging the movement of cells adhering to a substrate.[56–58] For this purpose the surface of the substrate is coated with a layer of colloidal gold nanoparticles (see Fig. 2d). Cells adhering to the substrate incorporate the Au particles. In this way cells migrating along the substrate leave behind a trail called a "phagokinetic track" in the nanoparticle layer. By imaging the particle layer with optical transmission microscopy or TEM a blueprint of the migration pathway of the cells is obtained. Compared to time lapse tracing of the migration of cells which requires online video microscopy of individual cells, phagokinetic tracks do not need to be recorded online. Many trails can be recorded in parallel on the same substrate and the trails can be imaged *ex situ*, as they consist of areas in the nanoparticle layer that are permanently free of nanoparticles. Although recently the same technique has also been introduced with both fluorescent quantum dots[59] and fluorescent latex beads, still gold is the predominantly used label for recording phagokinetic tracks.

4. Gold nanoparticles as a vehicle for delivery

Gold nanoparticles have been used for a long time for delivery of molecules into cells. For this purpose the molecules are adsorbed on the surface of the Au particles and the whole conjugate is introduced into the cells. Introduction into cells can either be forced as in the case of gene guns or achieved naturally by particle ingestion. Inside cells the molecules will eventually detach themselves from the Au particles.

4.1 Gene guns

The idea of gene guns is using Au particles as massive nanobullets for ballistic introduction of DNA into cells (see Fig. 3a).[60] DNA is adsorbed onto the surface of gold particles which are then shot into the cells. The ballistic acceleration of the gene-loaded micro- or nanoparticles is realized by different means like macroscopic bullets, gas pressure or electric discharges[61] and some types of guns are commercially available. Traditionally, gene guns have been used for the introduction of plasmid DNA into plant cells,[62,63] which results in expression of the corresponding proteins inside the cells. In this case ballistic introduction with massive particles is advantageous as it allows for traversing the rigid cell walls which surround the membranes of plant cells. However, gene guns are also used for delivery of DNA into animal cells, which do not possess cell walls.[64]

4.2 Uptake by cells

Cells naturally ingest colloidal nanoparticles[18] whereby particle incorporation can be specific (*via* receptor–ligand interaction) or nonspecific. The goal is again to transfer molecules which are adsorbed on the surface of the Au particles into the cells (see Fig. 3b). For specific uptake ligands specific to receptors on the cell membrane, such as transferrin which binds to membrane-bound transferrin receptors,[65–67] are conjugated to the surface of the gold particles. As specific uptake is more effective than nonspecific uptake, in this way ligand-modified Au particles are predominantly incorporated by cells which possess receptors for these ligands, but not by other cells. In this way, it is for example possible to direct particles specifically to cancer cells by conjugating them with ligands specific to receptors which are overexpressed on the surface of cancer cells but that are less present on healthy cells.[68] After incorporation nanoparticles are stored in endosomal/lysosomal vesicular structures inside

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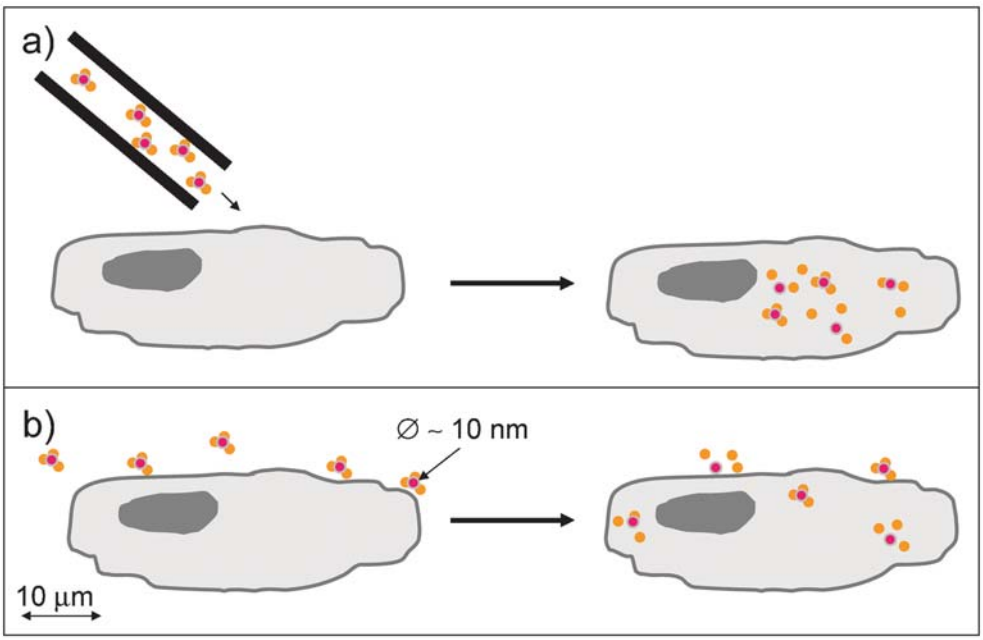

Fig. 3 Delivering with gold nanoparticles. Molecules (shown in orange) which are to be delivered inside cells are adsorbed on the surface of gold nanoparticles (core in red, stabilizing shell in grey). Once inside the cell these molecules will eventually detach themselves from the surface of the nanoparticles. (a) Gene guns: The nanoparticles are shot as a ballistic projectile into the cells using a so-called gene gun system. (b) Nanoparticle uptake by cells: The nanoparticles are either specifically or nonspecifically incorporated by cells. After ingestion the particles are stored in vesicular compartments inside the cells.

cells.[69] In order to release the particles from the vesicular structures to the cytosol their surface can be coated with membrane-disruptive peptides or the particles can be modified with peptides which allow for direct transfer across the cell membrane.[70–73] In this way it is possible to deliver molecules which are adsorbed on the surface of the Au particles upon particle incorporation inside the cells.[74,75]

Particle uptake-mediated delivery of molecules into cells is used mainly for two applications. First, in gene therapy DNA is introduced into cells, which subsequently causes the expression of the corresponding proteins.[76–78] Second, in drug targeting anti-cancer drugs are delivered specifically to cancer tissue.[68,79] Particle-mediated drug delivery by adsorbing molecules onto colloidal particles and transferring them into cells allows for delivery of molecules inside cells which would not have been ingested into the cells by themselves. This is based on the fact that colloidal nanoparticles are taken up by cells. Besides being loaded with the molecules to be delivered, particles can also be conjugated with ligands through which specific uptake by target cells can be facilitated. For such delivery applications no special property of gold particles is exploited, other than that they are small, colloidally stable, relatively easy to conjugate with ligands *via* thiol–gold bonds, and that they are inert and thus relatively biocompatible. Delivery applications using gold nanoparticles have been reviewed recently,[80] some similar strategies can in principle also be realized with colloidal silica, iron oxide, and organic polymer nanoparticles. Nevertheless, there are a few studies that already make use of the optical properties of Au nanoparticles for detection (which the other types of particles do not offer), after they have been ingested by cells.[81,82]

5. Gold nanoparticles as a heat source

When gold particles absorb light the free electrons in the gold particles are excited. Excitation at the plasmon resonance frequency causes a collective oscillation of the free electrons. Upon interaction between the electrons and the crystal lattice of the gold particles, the electrons relax and the thermal energy is transferred to the lattice. Subsequently the heat from the gold particles is dissipated into the surrounding environment.[83] Besides its combination with imaging techniques (see above in section 3), controlled heating of gold particles can be used in several ways for manipulating the surrounding tissues.[84]

5.1 Hyperthermia

Cells are very sensitive to small increases in temperature. Even temperature rises of a few degrees can lead to cell death. For human beings temperatures above 37 °C lead to fever and temperatures above 42 °C are lethal. This fact can be harnessed for anti-cancer therapy in a concept called hyperthermia. The idea is to direct colloidal nanoparticles to the cancerous tissue. This can be done by conjugating the particle surface with ligands that are specific to receptors overexpressed on cancer cells. The particles are then locally enriched in the cancerous tissue (either adherent to the cell membranes or inside the cells after internalization). If the particles can be heated by external stimuli then the temperature of cells close to the particles is raised and in this way cells in the vicinity of the particles can be selectively killed.[85] As mentioned above, Au particles can be heated by absorption of light, whereby the absorbed light energy is converted into

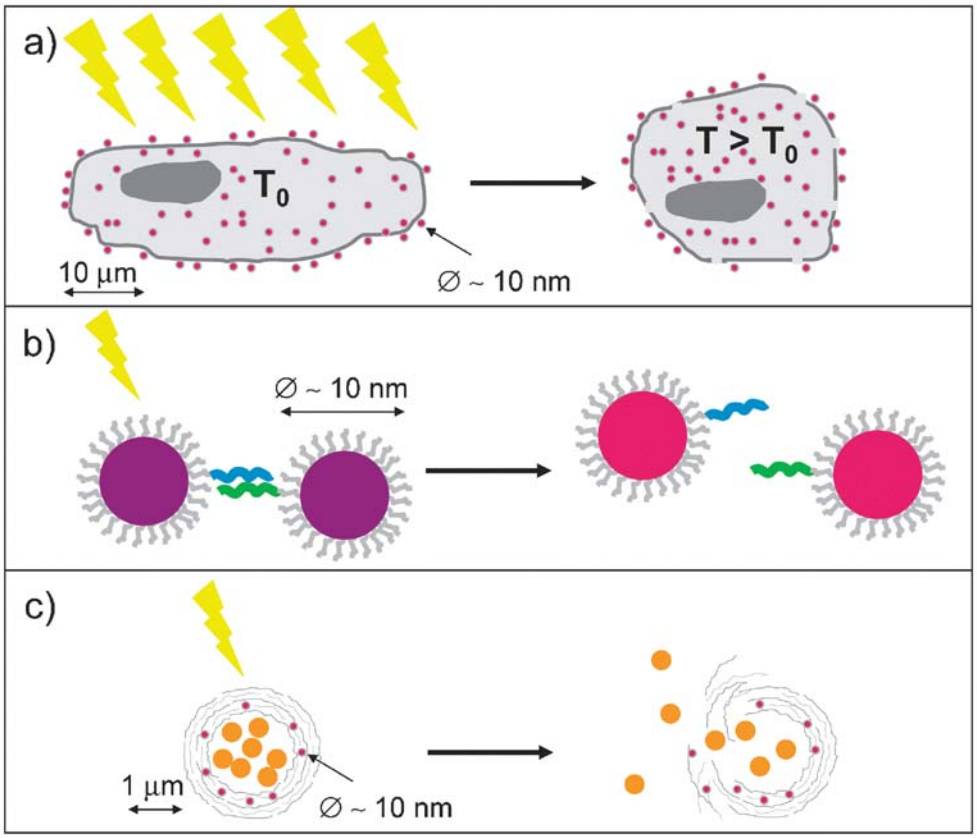

Fig. 4 Heating with gold nanoparticles. Gold particles (core in red, stabilizing shell in grey) are heated upon absorption of light (shown as yellow rays) and mediate the heat to their local environment. (a) Hyperthermia: The temperature inside cells (drawn in grey) is raised by illumination of gold particles. A temperature increase of only a few degrees is sufficient to kill cells. (b) Breaking of bonds: When gold nanoparticles (core in red, stabilizing shell in grey) are conjugated with ligands (shown in green) that are specific to receptors (shown in blue) which are bound to other gold particles, these two kinds of gold particles will be linked to assemblies mediated by receptor–ligand binding. As the distance between the particles in such aggregates is small, their plasmon resonance is shifted to higher wavelengths and the particle solution appears violet/blue. Upon illumination the gold particles get hot and the bonds of the receptor–ligand pairs melt. Therefore the assemblies are dissolved, the average distance between the particles is increased and the particle solution appears red. (c) Light-controlled opening of individual polymer capsules (drawn in grey) by local heating, mediated by Au nanoparticles. Gold nanoparticles are embedded in the walls of polyelectrolyte capsules. The capsule cavity is loaded with cargo molecules (drawn in orange). Upon illumination with light the heat created by the nanoparticles causes local ruptures in the capsule walls and thus release of the cargo.

thermal energy. Thus the idea is to enrich cancerous tissues with gold nanoparticles and to illuminate the tissue. Due to the heat mediated by the gold particles to the surrounding tissue, cancerous tissues can be destroyed locally (see Fig. 4a)[86–88] without exposing the entire organism to elevated temperatures.

Besides the general problem of local particle enrichment in the target tissue there is also a problem of principle involved. Tissue absorbs light in the visible region, and even infrared (IR) light can only penetrate relatively thin tissue. For this reason gold nanoparticles are needed which absorb light in the IR rather than in the visible range, such as gold rods or hollow structures.[89,90] Nevertheless hyperthermia by photo-induced heating of gold nanoparticles will work best for tissue close to the skin. For tissues deep inside the body heating with magnetic particles is favourable. Upon irradiating magnetic particles with radiofrequency (RF) fields, heat is generated by repetitive cycling of the magnetic hysteresis loop.[91,92] Compared with the optical excitation of Au nanoparticles at wavelengths of *ca.* 500–1000 nm, the excitation of magnetic nanoparticles works at lower frequencies (RF). As those are absorbed much less by normal tissue and thus penetrate deeper, particles inside the body can also be heated. On the other hand it is much more complicated to focus microwaves or radiofrequency waves than visible light and therefore photo-induced heating of gold particles is favourable for local heating of only small parts of tissue.

5.2 Optically triggered opening of bonds

Photo-induced heating of gold nanoparticles can also be used for the opening of chemical bonds (see Fig. 4b). The binding of complementary oligonucleotides (hybridization) to double stranded DNA, for example, is temperature dependent. Upon heating, double stranded DNA melts into two single strands. If DNA is linked to the surface of gold nanoparticles then local melting can be triggered by illuminating and thus heating the Au particles.[93,94] As light can be easily focused to a micrometre spot size a high degree of spatial control is possible

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which allows for very local heating. Similar concepts have also been used for the disassembly of protein aggregates by local heating.[95]

5.3 Opening of containers

Finally, photo-induced heating of gold nanoparticles can also be used for remotely controlled release of cargo molecules from containers (see Fig. 4c). This concept is based on embedding cargo molecules in containers, such as polymer capsules, whereby the walls of the containers are functionalized with gold nanoparticles. Upon optical excitation the gold nanoparticles are heated which causes local ruptures in the container walls and thus release of the cargo from the inside of the container.[96,97] Light-induced opening of polymer capsules can be performed on a single capsule level and it has already been demonstrated that upon photo-induced heating cargo molecules can be released from capsules inside living cells.[98] Moreover, cell membranes themselves can be perforated with the help of nanoparticles.[99]

6. Gold nanoparticles as sensors

Besides using gold nanoparticles as (passive) labels they can also be used for (active) sensor applications. Their aim in a sensor is to specifically register the presence of analyte molecules and to provide a read-out that indicates the concentration of the analyte. When an optical read-out is used, the presence of analyte can, for example, be indicated by changes in the optical properties of gold nanoparticles. Due to their small size, gold particle-based sensors could have an important impact in diagnostics.[82]

6.1 Surface plasmons

The plasmon resonance frequency is a very reliable intrinsic feature present in gold nanoparticles (with wavelengths around 510–530 nm for Au nanoparticles of around 4–40 nm diameter) that can be used for sensing.[100] The binding of molecules to the particle surface can change the plasmon resonance frequency directly,[101] which is observable by their scattered light in dark field microscopy, in particular on the single particle level. On the other hand the plasmon resonance frequency is dramatically changed when the average distance between Au particles is reduced so that they form small aggregates.[102] This effect of plasmon coupling can be used for colorimetric detection of analytes (see Fig. 5a). The method was pioneered by Mirkin and coworkers and is nowadays maybe the most well-known example of a gold-based sensor.[103–105] The original assay was developed for the detection of DNA. Gold nanoparticles are conjugated with oligonucleotides that are complementary to the target sequence which is to be detected. Without the presence of the target sequence the gold particles are freely dispersed and the colloidal solution appears red. In the presence of the target sequence the gold particles bind to the target by hybridization of complementary strands of DNA. As each gold nanoparticle is bearing several oligonucleotides, hybridization results in the formation of small aggregates of Au particles, which will lead to a change in the plasmon resonance and the colloidal solution appears a violet/blue colour. When the sample is heated, even single sequence mismatches result in a different melting temperature of the aggregates which causes colour change. Several DNA assays have been derived from this concept and nowadays the method is established in a way that quantitative detection of DNA sequences of very low concentrations is possible.[106]

The same concept can also be applied for analytes other than DNA. Gold particles can, for example, be connected by DNA in such a way that the average inter-particle distance is large enough to prevent changes in the plasmon resonance frequency. By using *e.g.* DNA sequences that change their conformation upon specific binding (such DNA-, RNA- or peptide-based sequences are called aptamers) of metals[107] or proteins,[108,109] the inter-particle distance is reduced and thus the colour of the gold colloids changes from red to violet/blue. Also enzyme activity can be monitored with such colorimetric assays, for example by the enzymatic biotinylation of nanoparticles and subsequent formation of aggregates with streptavidin-modified nanoparticles.[110] In the presence of an enzyme inhibitor, the first nanoparticles are not modified and no aggregation occurs.

Besides the detection of analytes, such colour changes can also be used to measure lengths. The concept of such ''rulers on the nanometre scale'' is again based on colour changes of gold particles if the gold particles are in close proximity. Different sites of a macromolecule can be linked to gold particles. By observing the colour of the gold particles the distance between these sites can be measured and in this way for example conformation changes in molecules can be observed.[111]

6.2 Fluorescence quenching

The fluorescence of many fluorophores is quenched when they are in close proximity to gold surfaces.[112–114] This effect can be used for several sensor strategies (see Fig. 5b). The first one is based on competitive displacement. For quantitative detection of a certain analyte, gold particles are conjugated with ligands that specifically bind to this analyte. Then the binding sites of the ligands are blocked by saturating them with analyte molecules (or molecules of similar structure that bind to the ligand), whereby these molecules are modified with fluorophores. As the fluorophores are in this way closely linked to the Au particles their fluorescence is quenched. After washing, these gold particles with blocked ligands are now added to the solution in which the concentration of the analyte should be detected. If no analyte is present there will be no fluorescence, as the fluorophores are quenched by the gold particles. Analyte molecules present in solution on the other hand will compete with the fluorophore-labelled analytes previously bound to the Au particles for the binding sites of the ligands on the Au surface.[16] In a continuous dynamic equilibrium analyte molecules in solution will displace analyte molecules bound to the ligands present on the particle surface. For reasons of simple statistics, the higher the concentration of analyte molecules in solution is, the fewer fluorophore-labelled prebound molecules will remain on the particle surface in equilibrium. This means that the higher the concentration of analyte molecules is, the more fluorophore-labelled molecules

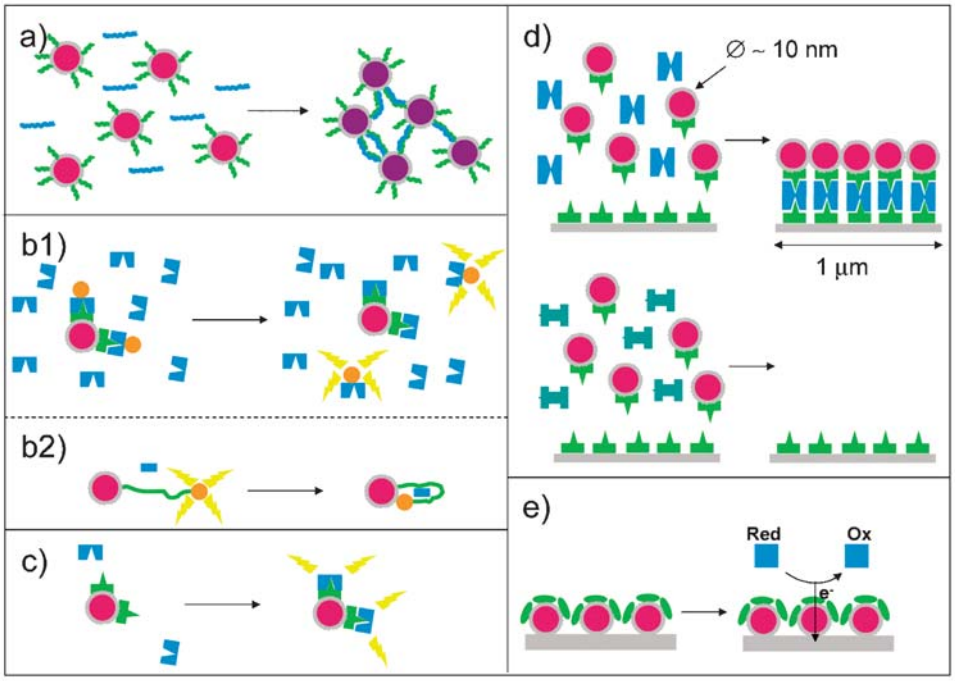

Fig. 5 Sensing with gold nanoparticles. For the specific detection of analytes (shown in blue) gold nanoparticles (core in red, ligand shell in grey) are conjugated with ligands (shown in green) that selectively bind to the analyte. (a) Colorimetric assays: Binding of the analyte to the ligands links several particles together to form small aggregates and the red colour of the colloidal gold solution shifts to purple/blue. (b) Quenching of fluorophores: (b1) Gold nanoparticles are conjugated with ligands that specifically bind to the analyte to be detected. The ligands on the nanoparticle surface are then saturated with molecules that bind to the ligands (shown in blue) and that have a fluorophore (drawn in orange) attached. As the fluorophores are in close proximity to the surface of the Au particles their fluorescence is quenched. The presence of analyte molecules competitively displaces part of the molecules with the fluorophores from the nanoparticle surface. As these fluorophores are no longer in contact with Au particles their fluorescence (symbolized as yellow rays) can be detected. (b2) Fluorophores (drawn in orange) are attached *via* linker molecules (drawn in green) to the surface of Au nanoparticles. Due to the length of the linker the distance between the fluorophore and the gold particles is big enough so that no quenching of the fluorophore occurs. Presence of the analyte (drawn in blue) changes the conformation of the linker molecules and as the fluorophores are now in close proximity to the Au surface their fluorescence is quenched. (c) Surface-enhanced Raman scattering: Gold nanoparticles are conjugated to ligand molecules which specifically bind to the analyte to be detected. The analyte (drawn in blue) in solution provides only a weak Raman signal. Upon binding of the analyte to the ligands present on the Au surface the analyte comes into close proximity to the gold particles and the Raman signal is dramatically enhanced (as symbolized by the yellow rays). (d) Gold stains: Ligands specific to the analyte to be detected are immobilized on a surface and conjugated to Au nanoparticles. Presence of the analyte (drawn in blue) causes the binding of the particles to the surface. Other molecules (drawn in dark green) do not cause binding of the particles to the surface and thus a washing step removes all gold particles. The presence of the analyte is then quantified by the number of Au particles bound to the surface. (e) Redox reactions: Redox enzymes (drawn in green) are conjugated to the surface of Au nanoparticles (core in red, ligand shell in grey) which are immobilized on top of an electrode (drawn in grey). The enzymes oxidize their present substrates (drawn in blue) from the reduced form to the oxidized one. The released electrons are transferred *via* the gold nanoparticles to the electrode, which can be measured as current.

will be released from the Au particle surface into solution and as there is no quenching in solution the higher the resulting fluorescence signal will be. A variation of this concept involves using Au nanoparticles as quenchers for quantum dots which are replaced by the analyte. When the Au nanoparticles are released, the fluorescence of the quantum dots increases.[115]

A second detection scheme works slightly differently. In this case a molecule is needed which changes its conformation upon binding of the analyte. This molecule is used as a spacer to link fluorophores to gold nanoparticles. Without the presence of the analyte the spacer molecule is extended and there is no quenching of the fluorescence of the fluorophore. Analyte molecules on the other hand will bind to the spacer which then changes its conformation in such a way that the attached fluorophore will be brought into close proximity to the Au surface, which results in quenching of the fluorescence. The higher the concentration of analyte in solution, the lower the recorded fluorescence signal will be. The same principle can be used in the opposite way when binding of the analyte stretches the spacer attached to the particles, and the quenched fluorescence increases after binding.[116–118] Due to dynamic binding and unbinding of the analytes to the ligand (spacer) molecules present on the Au surface, both of the above introduced sensor concepts are reversible. In contrast to fluorescence quenching by metal nanoparticles, it remains to note that there are also recent findings of metal-*enhanced* fluorescence and ideas about the exploitation of this effect for future applications.[119]

6.3 Surface-enhanced Raman scattering

Due to their characteristic spectra, many (macro) molecules can be detected by Raman scattering.[120] In Raman scattering the incident light is scattered with a low probability on

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vibrational and rotational states of the molecule. The scattering process is inelastic, and thus the scattered light can have a lower (Stokes, by depositing energy into the molecule) or higher energy (anti-Stokes, by gaining energy from the molecule) than the incident light. The energy shift is characteristic for the chemical structure where the scattering occurred and complex molecules have therefore a characteristic Raman spectrum that allows for detection and identification. While the scattering efficiency might depend on the wavelength of the incident light, the energy shift remains the same. Typically Raman signals are quite weak and therefore a sufficient analyte concentration is needed in order to provide enough signal. Raman scattering is dramatically enhanced if the molecules are close to a gold surface with very high curvature, as for example small gold nanoparticles. This effect is called surface-enhanced Raman scattering (SERS).[121–123] Due to the plasmon resonance of metal nanoparticles there is a strong enhancement of the electric field in close proximity to the particles, compared to the field strength of the incident light. This results in a much higher scatter probability and thus in a gain of several orders of magnitude of the Raman-scattered light intensity that is detected. SERS can be used for the detection of analytes (see Fig. 5c). Again the surface of Au nanoparticles is modified with ligands that can specifically bind the analyte. Upon binding to the Au particle the Raman signal of the analyte is dramatically enhanced and allows for its detection.[124,125] Recent developments include Au nanoparticles modified with Raman-active reporter molecules for the detection of DNA[126] or proteins,[127,128] and two-photon excitation.[129]

6.4 Gold stains

Instead of using fluorophores or absorbing dyes as read-out for ELISA-like assays (enzyme-linked immunosorbent assays) gold nanoparticles can also be used. The aim of such assays is the specific qualitative or quantitative detection of analytes, which is conceptually related to immunostaining (see section 3.1). For this purpose the analyte is immobilized on a surface, either by simple adsorption or specific binding *e.g.* by a capture antibody. Instead of enzyme-labelled antibodies, analyte-specific antibodies are conjugated to the surface of gold nanoparticles. The presence of analyte in the assay thus results in binding of gold particles to the surface (Fig. 5d).[130,131] The concentration of analyte molecules can be quantified by the optical absorption of the gold spot which is a function of the analyte concentration. Sensitivity can be increased by involving secondary antibodies and silver enhancement, where the gold nanoparticles catalyze the reduction of silver and are thus grown larger by a silver coating.[132] Similar assays using gold stains as read-out can also be applied for receptor–ligand systems without involving antibodies, such as the detection of target DNA sequences with complementary DNA.[133,134] In a similar way antibody-conjugated gold nanoparticles can be used for the detection of proteins or DNA after blotting the molecules from a gel onto a membrane.[135] For many years, gold nanoparticles conjugated with a variety of antibodies (''immunogold'') have been commercially available, and they are also used to enhance the contrast in electron microscopy (section 3.1). Alternatively, conjugated Au nanoparticles can be used to detect antigens (*e.g.* proteins[136] or DNA[137]) present on an electronic chip. The binding of the nanoparticles is read out by voltammetry, and again the Au nanoparticles can be enhanced by silver deposition in order to amplify the signal.

6.5 Electron transfer

Finally, gold nanoparticles can also be used for the transfer of electrons in redox reactions.[138] The idea of such assays is to detect analytes which are substrates to redox enzymes. The enzyme can specifically oxidize (or reduce) the analyte molecules (Fig. 5e). The flow of electrons released (or required) in this redox reaction can be measured as electrical current. For this purpose the enzyme is conjugated to the surface of the gold particles.[139] The enzyme–particle conjugates are then immobilized on the surface of an electrode (gold) which is connected to an amplifier for current detection, *e.g.* by cyclic voltammetry. Alternatively, the gold nanoparticles can be first immobilized on the electrode and then modified with enzymes.[140] In principle the enzyme could be directly immobilized on the flat gold electrode of the chip. However, the introduction of Au nanoparticles has several advantages. First an electrode covered with a layer of nanoparticles has a much higher surface roughness and thus larger surface area, which leads to higher currents. Second, because of the small curvature of small gold particles the contact of the Au particle with the enzyme can be more ''intimate'', *i.e.* located in close proximity to the reactive centre, which can facilitate the electron transport.[141,142]

7. Outlook

Colloidal Au nanoparticles possess a lot of interesting properties that make them useful for biological applications. Though similar applications can also be performed with colloidal nanoparticles of different materials, such as quantum dots, there are several features unique to gold particles. So far there is no indication of Au particle corrosion, and Au particles are inert, which makes them relatively biocompatible. Gold nanoparticles can be easily synthesized, they are colloidally stable, and they can be conjugated with biological molecules in a straightforward way. Due to their optical properties, in particular the surface plasmon resonance, they can be visualized with different methods and sensors based on changes of the plasmon resonance have been demonstrated. For this reason gold particles are now also used for different applications besides the ''classic'' examples of gene guns and immunostaining. Though in our opinion gold nanoparticles will never play a ''dominant'' role in biology we predict that they will be routinely used within several standard *in vitro* assays and kits and that there is still plenty of room for new research. In particular, we believe that the shift in plasmon resonance upon binding of molecules or changing the inter-particle distance will lead to a number of sensor assays for the detection of analytes which will become commercially available.

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