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FEATURE ARTICLE

Biofunctionalization of nanoparticles for cytosensing and cell surface carbohydrate assay

Lin Ding and Huangxian Ju*

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Developing sensitive and specific sensing strategies for probing the chemical and biological events in/at a living cell can ultimately contribute to the understanding of life processes. These strategies are also beneficial to early diagnosis of diseases for early and successful treatment. Biofunctionalization of nanoparticles can integrate remarkable properties of both biological components and nanoparticles, thus providing a promising platform for tracing the recognition and transduction processes and improving the sensing performance. This Feature Article reviews the current research regarding the biofunctionalization strategies, and the usage of the bionanocomposites in the arena of cytosensing and cell surface carbohydrate assay.

1. Introduction

The investigation on the spatiotemporal aspects of cellular components, biochemical processes, signaling and communication is critical for understanding cellular function.[1] This procedure needs sensitive and specific sensing strategies for probing the chemical and biological events in/at a living cell. These strategies are also beneficial to finding new specific biomarkers and monitoring of biomarkers for early diagnosis and treatment of diseases, particularly malignant tumors.[2–5] Thus the development of novel sensitive and specific sensing strategies has become an attractive field in life science, biosensing and nanobiotechnology.

To understand cellular function, researchers need to track biomolecules in their native environments, which can be achieved by specific recognition between biomolecules, the target, and the probe. Biofunctionalization of nanoparticles (NPs) provides inexhaustible avenues for preparation of specific probes. The newly designed probes have extensively been used for development of efficient sensing systems, which consist of recognition elements and transduction process. The biomolecules used for biofunctionalization play a key role in the specific recognition, while the nanoparticles can amplify the recognition events and improve the transduction process, thus greatly enhancing the

State Key Laboratory of Analytical Chemistry for Life Science, Department of Chemistry, Nanjing University, Nanjing, 210093, P.R. China. E-mail: hxju@nju.edu.cn

Lin Ding

Lin Ding graduated with a B.S. degree in chemistry from Nanjing University in 2004. She then started a project on the electrochemical detection of tumor cells and cell surface glycans under the supervision of Prof. Huangxian Ju at Nanjing University, where she obtained her Ph.D. in 2009. She is now a lecturer in Department of Chemistry, Nanjing University.

Huangxian Ju

Huangxian Ju received B.S., M.S. and Ph.D. degrees from Nanjing University in 1986, 1989 and 1992, and became a full professor in 1999. He is a chief scientist of 973 Program, Changjiang Chair Professor, and the member of editorial boards of 15 international journals and 9 key Chinese journals. His research interests focus on analytical biochemistry and nanobiotechnology. He has published 320 papers in SCI journals, which have been cited more than 7450 times with an H-index of 48.

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sensing signals. In recent years this technology has been applied in cytosensing and monitoring of biomolecules in/at living cells.

In order to improve the performance of cytosensing, the exploitation of functional nanostructures with high biocompatibility and stability, low toxicity, and specificity of targeting to desired cells or cellular receptors has emerged as a cornerstone solution. NPs often show broad and tunable optical, magnetic, electrical and photothermal properties with large surface-to-volume ratio, which allows for the integration of different functions into one single entity.[6–9] These functions have been proven to be vital tools in a diverse range of cellular imaging, sensing and drug delivery applications.[10,11] The NPs used in cytosensing systems include metal NPs,[12–14] quantum dots (QDs),[15,16] silica NPs[7,17] and paramagnetic NPs.[8,18,19] These NPs can be fashioned with a wide range of biological-related small ligands and large biomacromolecules through chemical surface modification and conjugation.[20–22] The resulting biofunctionalized NPs possess the unique properties of their constituent materials,[23–25] thus providing a promising platform for sensitive, selective and reliable cytosensing.[26,27]

This Feature Article provides an overview of the recent advances in biofunctionalization of NPs for construction of sensing platforms toward cell-relevant species by discussing how the biofunctionalized NPs are being integrated with various detection technologies. Different from a mass of review articles dealing with NP-based optical imaging, this review focuses on the application of NPs in cytosensing, including electrochemical, magnetic, colorimetric, chemiluminescent and visual detection. Some newly developed optical imaging methods using biofunctional NPs are also involved. The surface modification of NPs and conjugation of biomolecules or biocompatible molecules with NPs have been comprehensively discussed. Several modern technologies and instrumentations, as the classification criteria of the cytosensing application, have been reviewed. As an example of cytosensing applications in probing the biological processes of cell surface carbohydrates, this review also gives a brief introduction to the application of biofunctionalized NPs in cell surface carbohydrate assay.

2. Biofunctionalization techniques of NPs

In NP-based cytosensing, the solubility, stability, cytocompatibility and recognition/target ability of NPs in aqueous media are of major concern. Biofunctionalization of NPs, which means the attachment of biomolecules (such as small molecules, peptides, aptamers or antibodies) or biocompatible molecules (such as poly(ethylene glycol) (PEG)) to NPs, leads to the incorporation of unique biological-adaptable features to NPs. Great efforts have been devoted to the development of techniques for conjugating biomolecules with NPs. The two main kinds of methods are post-conjugation and one-pot synthesis. Post-conjugation of molecules on NPs indicates that the conjugation step follow the NP synthesis step, while one-pot synthesis means that the biomolecules are tethered to NPs during the formation of NPs. The choice of functionalization strategies should accord to the properties of both NPs and biomolecules. The maintenance of the biological activity is vital to the design of modification procedure.[17] Most importantly, the aggregation of NPs and the nonspecific binding of other species on NPs should be suppressed or avoided.[17] In addition, introducing multiple functionalities through surface modification and biofunctionalization is of great value for cytosensing, as it provides more flexibility for multiplexing detection and multifunctional applications (such as drug delivery, disease therapy, gene interference, *etc.*).[13]

2.1 Biomolecules and biocompatible molecules for functionalization of NPs

In order to achieve the biological function of NPs during cytosensing, various kinds of biomolecules or biocompatible molecules have been used for modification of NPs according to the customized function, such as increasing water solubility, enhancing stability, achieving cytocompatibility, affording recognition capability and signal amplification ability. In some cases, a few molecules can perform several functions simultaneously.

One of the major factors in producing NPs for cellular applications is their water solubility. Silica,[28] poly(amino acid)[29] and PEG-phospholipid[11,30] coatings have been used to enhance water solubility of hydrophobic NPs. Importantly, the content and chain length of PEG can be tailored to the requirements of the application.[31] Phosphatidylcholine is often used in conjunction with PEG-phospholipids to control the density of the PEG chains on the particle surface.[11]

The stability of NPs is of upmost importance for cytosensing. Charged phospholipids experience repulsive forces between micelles, which is useful in decreasing aggregation. Proteins, such as bovine serum albumin (BSA), can be linked on NPs to withstand high salt concentration. BSA can also be used to encapsulate Raman signature molecule-conjugated gold NPs (AuNPs) for preventing their aggregation and desorption. In this case, BSA simultaneously acts to cross link amine-containing antibodies with glutaraldehyde.[32]

Biocompatible polymers, such as dextran and chitosan, have often been used for coating NPs to provide them with colloidal stability.[18,33] However, these polymeric coatings are typically bound loosely to the surface of NPs. PEG moieties, in some cases appended with dihydrolipoic acid (DHLA),[34] can be assembled tightly on the surface of NPs for providing good stability in a wide pH range. The PEG moieties also provide the option of a wide range of terminal functional groups for bioconjugation and covalent modifications.[34,35] The PEGylated ligand may allow the conjugation of the Zn-rich surface of QDs with polyhistidine-tagged proteins simultaneously.[34,36,37]

The PEG polymer with long chain length substantially increases the hydrodynamic size of NPs, which hinders the conjugation of larger and globular protein on NPs, and prevents renal elimination in *in vivo* applications. In order to overcome these shortcomings, recently, small DHLA-based ligands with extended pH and salt stability have been designed.[35,38] These ligands rely on an inherent zwitterionic nature, and can increase the chance that oligohistidine (His)-tags on proteins reach the QD surface to form stable binding.[38]

To enhance the cytocompatibility, natural polysaccharides, such as chitosan,[39,40] have been used to coat or immobilize NPs on electrodes to increase cytocompatibility during electrochemical and electrochemiluminescent cytosensing. Encapsulation of NPs in phospholipid micelles bestows favorable

biocompatibility properties upon the encapsulated particles,[41] and they are known to have low critical micelle concentrations and high kinetic stabilities. The PEG on the NP surface can improve the cytocompatibility, blood circulation time and internalization efficiency of the NPs.[42]

BSA and PEG are commonly used to reduce the non-specific interactions. In the case of using PEG, the ethylene glycol chains can function as a shielding component to minimize nonspecific binding.[17] Glutathione, an abundant triamino acid peptide in nature, can also be used as a surface ligand due to its low affinity to serum proteins and the capability of minimizing nonspecific protein adsorption on the NPs.[43,44] Carbohydrate has been used to coat magnetic NPs (MNPs) to suppress the nonspecific sorption of serum proteins.[45]

Different types of enzymes have been conjugated on NPs for signal amplification by their enzymatic catalysis ability.[46,47] In this case, enzymatic substrates are often required to be added in detection solution.

To achieve the active cell targeting,[6] different kinds of recognition molecules that can bind specifically to cells have been attached on NPs. These recognition molecules include antibodies,[29,32,48,49] small molecules,[28,50–53] peptides,[37,38,54–56] aptamers,[57–59] carbohydrates,[42,60,61] proteins,[34] and lectin.[46,62,63]

In principle, the link of antibodies to NPs can increase the specificity for cellular receptors.[64] However, this technique shows several limitations: 1) it is nontrivial to control the orientation and the number of antibodies on NPs; 2) it is not practical to use antibodies of relatively large size ($\geq$150 kD) for live cell labeling of intracellular structures because of the cell impermeability; 3) antibodies have a high propensity to cross-react with each other, which increases the difficulties for multi-labeling a particular set of cellular targets simultaneously;[54] and 4) the selectivity of antibodies towards different cell types is limited due to the fact that antigens used for cell recognition are normally not exclusively expressed on any single cell type.[65] Thus, the exploitation of 1) single-chain variable fragment (scFv) antibodies with smaller size, 2) small-molecule ligand/receptor pairs, 3) various peptides, and 4) specific aptamers have already gained popularity.

To reduce the steric hindrance toward recognition for cells, the scFv antibodies have been used to replace full-size antibodies to perform the recognition function, which has been demonstrated to not only maintain the recognition properties but also improve the detection sensitivity of the receptor on cancer cells.[32,48]

Several reported ligand/receptor pairs include folic acid/folate receptors,[28,50,53] biotin/avidin,[34] glutathione S transferase/glutathione[66,67] and trimethoprim (TMP)/Escherichia coli dihydrofolate reductase (eDHFR),[52] *etc.* Folic acid (FA) and methotrexate have been used to graft silica-coated CdSeS QDs[28] and superparamagnetic NPs[50] to recognize cellular folate receptors. However, the cytotoxicity of methotrexate hampered its usage. The decoration of the coordination complex of nickel-nitrilotriacetic acid (Ni-NTA) on NPs, *via* a dopamine or other linker, can achieve high specificity and capacity.[68] The potential cytotoxicity associated with nickel, however, prevents the direct use of Ni-NTA on live cells for long duration.[52] Recently, Xu's group[52] has demonstrated the first example of NPs that selectively bind to eDHFR by decorating TMP on iron oxide NPs. The TMP/eDHFR pair is considered to be a bio-orthogonal system in eukaryotic cells.[52] The binding between TMP and eDHFR is monovalent, which disfavors the cross-linking that usually occurs in the biotin/avidin-based systems. Thus the conjugate of TMP-NPs provides a biocompatible, convenient and versatile platform for investigating the cellular responses to perturbation of proteins.[52]

Many mammalian cells overexpress receptors of some kinds of proteins (such as transferrin lactoferrin, transforming growth factor-α, ceruloplasmin, elastin, albumin, insulin and growth factors) on their surface. Thus these proteins have been used to conjugate NPs to mediate the specific recognition by cells. He *et al.*[69] have reported the transferrin-mediated uptake of AuNPs by tumor cells.

The use of peptides for endowing target capability to NPs possesses advantages that lead to significant reduction of expenses associated with synthesis and the ultimate nanoconjugate size.[70] Additionally, covalent conjugation of multiple peptides to one NP may considerably increase the binding capacity of the resulting complexes to the target through multivalent interactions. Commonly used peptides for cellular recognition include ones toward G protein-coupled receptors, integrins, and ion channels.[71]

Decorating NPs with cell-penetrating peptides is a popular way to achieve internalization by cells.[38,54] Canonical polyarginine peptidyl motifs have been demonstrated to facilitate endocytic uptake. Based on the N-terminal oligoarginine sequence, a cell penetrating peptide sequence (CPP = R_9GGLA (Aib)SGWKH_6) composed of two functional sequences that are separated by a GGLA(Aib)SGWK linker has been designed for delivery of QDs.[38] Peptide also provides a solution for simultaneous and multicolor labeling of distinct intra- and extracellular compartments/structures in a live cellular system.[54]

In addition, peptides have been demonstrated to be useful to adjust the cell membrane adsorption ability of NPs. In a recent study by Zheng's group,[43] a natural peptide, glutathione was used along with cysteamine as surface ligand to create a class of $\sim$3 nm luminescent AuNPs. The prepared NPs show little interaction with serum proteins and pH-dependent adsorption onto live cell membranes in a biological pH range from mildly acidic to neutral (5.3–7.4). This pH-dependent membrane adsorption ability is attributed to the prevention of glutathione against nonspecific protein adsorption and the binding of positively charged cysteamine to the negatively charged cell membrane through electrostatic interaction.

Aptamers are single-stranded DNA or RNA molecules created *in vitro* through systematic evolution of ligands by exponential enrichment (SELEX) for the recognition of target analytes with high specificity. Aptamers possess a low molecular weight, exhibit easy and reproducible synthesis, easy modification, fast tissue penetration, low toxicity or immunogenicity, easy storage, and high binding affinity and selectivity.[59] Recently, more and more attention has been given to the development of aptamer-AuNP probes for detection of cancer cells.[57] However, some reported aptamers show weak binding affinity and low signal in molecular imaging, which limits their ability for highly sensitive detection of cancer cells. One solution for this problem is the combined utilization of aptamer and antibody.[65]

Carbohydrates are involved as recognition markers in numerous biological processes, mainly occurring on the surfaces

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of cells. They can be recognized by carbohydrate-binding proteins, such as antibodies, enzymes, and lectins. Metal NPs have been used to tether a variety of carbohydrate ligands.[42,60,61] The coating of carbohydrate to NPs is mainly achieved by two different routes, *in situ* coating and post-synthesis coating. In the case of *in situ* coating, the precipitation of NPs is done in the presence of polysaccharides.[39,72] For the post-synthesis case, carbohydrates are often modified with functional groups, which can be directly conjugated to the NP surface during ligand exchange[61] or linked to the functional groups exposed on the modification layer of prepared NPs.[42] For example, very recently, sugar-coated iron oxide NPs were prepared by coating the NPs with organic ligands bearing a phosphonate group on one side and rhamnose, mannose, or ribose moieties on the other side through ligand exchange, due to covalent bonding between the Fe^{3+} atom and the phosphonate group.[61] In another study, thiol-modified carbohydrate was linked to the terminal amine of PEG-NH_2 conjugated on QD using 4-maleimidopropanoic acid *N*-hydroxysuccinimide (NHS) ester as a linker.[42]

2.2 Functionalization techniques for NPs

A ligand layer is normally assembled around the NP core to protect NPs from aggregation and endow NPs with novel physical, chemical and even biological properties. This layer may be comprised of small molecular ligands, amphiphilic polymeric ligands or dendrimeric materials.[53] Post-conjugation ways of biomolecules on the ligand layer assembled NPs depend on the physical properties and functional groups presented by the ligand. In some cases, prior to biofunctionalization, chemical modification[34,50] or ligand exchange[54,61] is a critical step in using NPs for cytosensing. With these treatments, the favorite functional groups (*e.g.*, carboxyl, amine) can be generated on the surface of NPs, which makes the NPs water-soluble. Self-assembled monolayers (SAMs) with different functional groups are widely adopted for modification of the NP surface.[8,73] The direct assembly of a mixed monolayer of hydrophilic PEG and a functional ligand for further conjugation has been suggested.[74] Here, the PEG chains act as a shielding component to minimize nonspecific binding. Afterwards, biomolecules are usually attached to NPs by noncovalent attachment, covalent binding or affinity interaction.[13,17,19,75]

2.2.1 Non-covalent attachment. For non-covalent attachment, electrostatic interaction or physical adsorption is a rather simple process.[13,75] In cytosensing direct adsorption of proteins with recognition ability on AuNPs is widely used for preparation of AuNP-based probe.[46,63] However, either stability or durability of the functionalized NPs may be problematic. Owing to the inherent characteristic of the interaction, the nonspecific adsorption between the AuNP probes and other species in the cell sample also needs to be avoided.

Encapsulation of NPs by biomolecules can prevent aggregation of the NPs and endow NPs with some biological function. These biomolecules are mainly biocompatible natural polymers, such as chitosan,[39] dextran, gelatin, polypeptide, cross-linked albumin. Synthetic polymers, such as PEG, are also used to encapsulate NPs.[19]

2.2.2 Covalent attachment. The covalent modification of NPs offers high stability and has been demonstrated to be quite robust. This technique is normally more complex, and sometimes requires intensive synthesis. Some cross-linkers such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS), *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (SMCC) and *N*,*N*′-methylenebisacrylamide (MBA) are usually used to covalently link biomolecules to the functional groups of monolayer assembled on NPs. The limitation of covalent immobilization of proteins using primary amines is their random orientation on the NP surface.[17]

In particular, the most common technique used for immobilization of ligands to AuNP or QD surfaces is the covalent linkage of thiol or disulfide.[12,13,76] His_n-based affinity interaction has also been used to conjugate His_n-appended proteins and peptides on Zn-rich surfaces of QDs for cellular probing applications.[34,36–38] His_n-interactions arise from collective metal-affinity coordination between the pendant imidazolium side-chains of histidine residues and the Zn-rich surface of QDs.[38] The advantages of this method lie in the rapid self-assembly with an intrinsically high affinity, controllable biomolecular orientation on the QD, minimal effects on the tertiary structure and biological activity of the appended proteins and peptides.[38]

2.2.3 Affinity interaction. Functionalization of NPs with groups that provide affinity binding ability is a versatile method for tethering biomolecules specifically. Biotin-avidin/streptavidin is the most widely used recognition pair, owing to the high binding constant ($K_a = 10^{15}\ M^{-1}$).[77] The bond formation between biotin and avidin/streptavidin is very rapid, and once formed, is unaffected by most extremes of pH and temperature, by organic solvents and by other denaturing agents.[17] Besides, protein A conjugate bound to NPs can be used as a versatile linker to Fc fragments of various immunoglobulins,[13] and carbohydrate modified NPs can be used to immobilize their respective binding lectins.[40] Recently, a very specific and strong noncovalent interaction ($K_a \sim 10^{14}\ M^{-1}$) between two proteins from *Bacillus amyloliquefaciens*—barnase and barstar—has been used for assembly of nanocomposites comprised of antibody and different NPs to achieve cell-specific targeting.[78] The major advantages of this pair include: 1) it is bio-orthogonal to mammalian cells; 2) the small size of barnase (12 kDa) and barstar (10 kDa) suggests lower immunogenicity than that of larger proteins like streptavidin; and 3) the two proteins can be linked to antibodies by recombinant protein techniques. Thus it is particularly suitable with *in vivo* applications.[78]

2.2.4 Flexible assembly of conjugation strategies. In some cases, biomolecules are conjugated to NPs through a combination of the above-mentioned strategies, and flexible assembly of these conjugation strategies can endow NPs with different biofunctions. For example, in a study by Liu *et al.*,[34] the surface of QDs was modified with heterobifunctional ligands incorporating dihydrolipoic acid, a short PEG spacer, and an amine or carboxylate terminus. These QDs were amenable to covalent modification of streptavidin *via* simple carbodiimide coupling chemistry. Furthermore, the QDs could be further given dual

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functionality by simultaneous covalent attachment of a rhodamine Förster resonance energy transfer (FRET) acceptor to the ligand and binding of polyhistidine-tagged streptavidin on the same nanocrystal to create a targeted QD, which exhibited dual-wavelength emission.

2.2.5 One-pot synthesis of biofunctionalized NPs.

NPs can be biofunctionalized with DNA,[76] protein,[27,79] or carbohydrate[39,45,72] during their synthesis process, including QDs,[76] MNPs,[45] AuNPs,[39,72] Au clusters[27,79] *etc.* The biomolecules added in the system play different roles, for example reducing metal ions, stabilizing NPs, programming NP growth and target function, guiding assembly of NPs. A chimeric DNA molecule ligand, designed with a ligand domain using phosphorothioate as backbone and a recognition domain using phosphate as backbone, has been used for programmed and simultaneous synthesis and biofunctionalization of CdTe QDs for specific binding to nucleic acids, protein and cell targets.[76] Co-precipitation of Fe(II) and Fe(III) salts with ammonium hydroxide in the presence of mannose solution can produce very fine MNPs of 2 nm with a mannose coating, which suppresses the nonspecific sorption of serum proteins.[45]

Using proteins to induce the synthesis of NPs has recently gain increasing attention due to the advantages: 1) proteins can act as green-chemical reducing and stabilizing agents, 2) their complex 3D structures can withstand a wide range of pH conditions, 3) the associated biological role of protein was retained, and can be pursued during cytosensing. For example, apoferritin has been used to guide controllable Au cluster formation at the ferroxidase enzymatic center of the heavy chain of ferritin.[27] Insulin has also been used as a template to direct the growth of fluorescent Au nanoclusters, which can recognize insulin receptor overexpressed on the differentiated C2C12 cells.[79]

2.3 Cytotoxicity of NPs

The cytotoxicity of biofunctionalized NPs has become a public concern in cytosensing.[80] For example, the potential cytotoxicity of QDs, because of the toxic heavy metals (*e.g.*, Cd, Hg, Pb, and Zn) included in the core, has been a major impediment to their widespread application.[80–82] Therefore, it has become critical to develop nontoxic and biocompatible NPs and fully understand the interactions between NPs and living cells.

The studies of toxicity are usually based on the interactions between NPs and cells, including their uptake by cells. Early studies have suggested that the release of core components, the generation of reactive oxygen species (ROS), and nonspecific binding to cellular membranes and intracellular proteins are the major mechanisms of the observed cytotoxic effects of QDs.[83,84] Recent data have suggested that the induction of autophagy by certain sizes of NPs plays an important role in their toxic actions.[85] Interestingly, the chirality of biomolecules for modification of QDs has also been demonstrated to induce autophagy and cytotoxicity.[85] This result highlights the important role of the conformation of the stabilizers, and provides important insight for the design of more "inert" surface coatings for QDs.

3. Biofunctionalized NPs for cytosensing

Cytosensing of the biochemistry and biophysics of living cells has been of prime interest for decades. Numerous approaches have been proposed to achieve real-time, noninvasive analysis of chemical and physical properties at unperturbed cellular physiological status. Significant progress is based on the integration of biofunctionalized NPs into the sensing scheme design. Here the application of biofunctionalized NPs in cytosensing is classified into four types according to detection techniques: 1) optical cytosensing; 2) magnetic cytosensing; 3) colorimetric cytosensing, and 4) electrochemical cytosensing.

3.1 Biofunctionalized NPs for optical cytosensing

Optical detection is the most widely used method for sensing and imaging of biological systems. According to the luminescent mechanism, it can be divided into three categories: 1) fluorescence; 2) surface-enhanced Raman scattering (SERS) and 3) chemiluminescence (CL).

3.1.1 Biofunctionalized NPs for fluorescent cytosensing.

Fluorescence detection provides an indispensable way to locate, monitor and track biological targets and biochemical processes on cell surfaces or within intracellular environments.[86] However, the limited types of detection probes restrict the application of cellular fluorescent sensing. In cellular concept, the probes should meet the following conditions: 1) such probes should be cytocompatible, and stable in cell media; 2) they can be used to achieve real-time, noninvasive analysis of chemical and physical properties at unperturbed cellular physiological status; 3) they can be conjugated with targeting factors towards specific cells or components of cells; 4) optical signal transduction mechanisms should be constructed based on the probes;[87,88] and 5) given the complex biochemical milieu in a physiological solution, the optical readout ideally should be ratiometric.[89]

Owing to the unique optical properties, luminescent NPs, including intrinsically luminescent nanomaterials and luminescent species-doped NPs, have already been widely used for diverse applications. Functionalization of luminescent NPs with biomolecules can lead to powerful and cytocompatible nanoprobes for cytosensing.

3.1.1.1 Biofunctionalized QDs for fluorescent cytosensing. Within the field of fluorescence-based cellular assay, QDs have received a great deal of interest due to their bright and stable fluorescence. QDs, consisting of an inorganic core surrounded by an organic shell, have various photophysical characteristics, including size-tunable narrow-symmetric photoluminescence (PL) spanning from the UV to near-IR, high QYs, large effective Stokes shifts, strong chemical/photostability, some of the largest two-photon action cross sections available and the ability to excite multiple QD populations at one wavelength.[16,54,90] These properties have made them useful as FRET donors, thus they can be used for fluorescent signal transduction of cytosensors.

Based on pH-dependent fluctuation of absolute fluorescence intensity, QDs have been used for pH sensing in human ovarian cancer cells.[91] A key limitation of the approach is that the QD fluorescence is not only sensitive to pH but also affected by other

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compounds in solution. Therefore, for complex samples, it is better to distinguish the pH effect from other factors.[92]

For design of *in vivo* QD-based FRET cytosensing systems, the size of the NPs is a major concern, because the final size of a water-soluble, functionalized QD should not exceed the donor–acceptor distance required for efficient FRET. Thus, small QDs are more suitable for highly specific, efficient, and sensitive fluorescent cytosensing.[90] In addition, peptides and aptamers, which are much smaller than antibodies, are better choices as recognition components to functionalize QDs.[54]

Recently, as an exciting advance in the field of nanomedicine, QDs have been engineered to carry distinct classes of agents for simultaneous cellular sensing, imaging and therapeutic applications.[93,94] A novel QD-aptamer (Apt)-doxorubicin (Dox) conjugate [QD-Apt(Dox)] has been developed by Bagalkot *et al.*[93] as a multifunctional NP system for targeted cancer imaging, therapy, and sensing. A10 RNA aptamer, which recognizes the extracellular domain of the prostate specific membrane antigen (PSMA), was used to functionalize QD, and the Dox, a fluorescent anthracycline drug emitting at 520–640 nm, was then intercalated into the double-stranded CG sequences in the PSMA aptamer. The formation of the conjugate led to a Bi-FRET mechanism, which included a donor–acceptor model FRET between QD and Dox and a donor–quencher model FRET between Dox and aptamer. As shown in Fig. 1, after specific uptake of QD-Apt(Dox) conjugates into the target cancer cell through PSMA mediated endocytosis, the release of Dox from the conjugates could induce fluorescence recovery of both QDs and Dox in cells, leading to a cancer imaging, therapy and sensing nanoparticle conjugate system *in vitro*. This strategy was sensitive enough to detect cancer at a single cell level since both QDs and Dox gave sharp images of cancer cells with low background noise.

Other mechanisms have also been used for fabrication of cytosensors. For example, the coupling system of negatively capped CdSe/ZnS QDs with an oxidized cytochrome c (Cyt c) has been demonstrated for fluorescent sensing of a superoxide radical ($O_2^{\cdot}$) in living cells.[95] Oxidized Cyt c can efficiently quench the fluorescence of QDs, whereas the reduced form of Cyt c leads to the recovery of the fluorescence of the QDs. Based on the temperature sensitive character of QDs, QD nanothermometers have been developed for revealing heterogeneous local thermogenesis in living cells.[96]

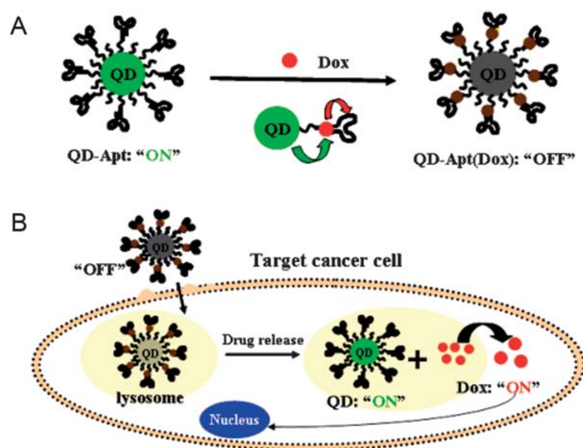

Fig. 1 (A) Schematic illustration of QD-Apt(Dox) Bi-FRET system. (B) Schematic illustration of specific uptake of QD-Apt(Dox) conjugates into a target cancer cell through PSMA mediate endocytosis. (Adapted with permission from ref. 93. Copyright 2007 American Chemical Society.)

The main limitation of using QDs for cytosensing lies in the toxic constituent elements like cadmium. To eliminate such a possible drawback, the encapsulation of QDs within a benign matrix, such as silica[97] and amphiphilic polymers has been used.[31] Recent work has employed the combination of silica and amphiphilic polymer to stabilize CdSe/ZnS QDs in a broad range of chemical conditions including strong acidic solutions, which is unavailable for any of the current encapsulation technologies used alone.[92] Besides, the drawbacks of QDs such as photoblinking, and the presence of nonradiant dark particles, also limit their applicability.[98] In recent years, several alternative types of luminescent NPs have been introduced for cytosensing.

3.1.1.2 Biofunctionalized polymer NPs for fluorescent cytosensing. As an alternative, exploiting polymer NPs are of ongoing interest as reliable fluorescent probes. These nanoprobes can be divided to two main types according to nature of the luminescent species (extrinsic or intrinsic): 1) fluorescent dye doped polymer NPs and 2) luminescent semiconducting polymer NPs (SPNs).

1) Fluorescent dye doped polymer NPs.
This type of polymer NPs is based on the encapsulation of dye molecules within a biocompatible, nonfluorescent polymer matrix. In comparison to fluorescent dyes, these nanoprobes are much brighter because many dye molecules can be embedded inside a single NP, allowing for signal amplification. As a pioneering work, Kopelman and coworkers[99] have developed a series of polymer NP sensors named PEBBLE (Photonic Explorer for Biomedical use with Biologically Localized Embedding). Different types of fluorescent analyte responsive indicators have been trapped within various polymeric nanoparticle matrixes such as polyacrylamide, liposome and polymer-capped stabilized liposomes[87] by various methods, including encapsulation, covalent linkage, physical adsorption, *etc.*[100,101]

In addition to high brightness, NP-encapsulated fluorescent probes provide a platform for incorporating more than two types of dyes (*e.g.*, one analyte-responsive indicator and one analyte insensitive dye for use as an internal reference) within a single matrix for achieving ratiometric fluorescence detection. Ratiometric measurement has the advantage that it is minimally affected by the fluctuation of the excitation source, probe concentration, photobleaching of probes, or any drifts in the instrument or the environment.[1]

Recently, due to the stability, biocompatibility and softness, hydrogel NPs (nanogels), colloidally stable NPs made from hydrogels, are appealing probes for use in chemical and biochemical sensing.[102] Fluorescent nanogels have been reported to sense temperature and pH in the cytoplasm of living cells.[103] In a later work by Wolfbeis's group,[104] a ratiometric fluorescent nanogel has been prepared rather simply from an inert but biocompatible polyurethane polymer that is pH sensitive by loading it with the pH indicator bromothymol blue. Furthermore, efficient FRET inside the nanogel is achieved by addition of two standard fluorophores coumarin 6 and Nile red. This

nanogel is capable of intracellular sensing of pH values in the physiological range. Through replacing the respective indicator dyes (probes) by indicators for other ions, various kinds of sensing nanogels can be constructed, and are likely to have a wide scope in terms of intracellular chemical sensing.

2) Semiconducting polymer NPs.

A major challenge associated with the above-mentioned polymer NPs is the leaching of the embedded dyes from the matrixes, and the use of an optically inert matrix which limits the potential brightness and optical functionality of the final NP probe. The limitation has been addressed by the emerging use of SPNs for cytosensing. It has been demonstrated that SPNs show favorable properties, such as exceptional fluorescence brightness (tens of times better than that of quantum dots), fast radiative rates, high photostability, large two-photon action cross sections, ease of processing and low cellular toxicity,[11,105,106] thus meeting the demands for cytosensing. However, bioconjugation and controlling the surface chemistry of SPNs is a challenge. Recently several methods have been developed for conjugating a wide range of biomolecules to SPNs.[11,89,105,107,108] Similar to dye-doped polymer NPs, SPNs also provide a versatile platform for fabrication of FRET-based ratiometric cytosensors for intracellular physical parameter measurement (such as pH[1] and temperature[89]). An important issue should be pointed out that the cytocompatibility of these nanoprobes should be further investigated in future.

3.1.1.3 Biofunctionalized AuNPs for fluorescent cytosensing. Frequently used colloidal AuNPs do not exhibit fluorescence; on the contrary, they are effective fluorescence quenchers. Thus AuNP-based fluorescent cytosensing can be designed as a quenching assay. For example, a homogeneous competitive strategy has been developed utilizing fluorescence quenching for quantification of eukaryotic cells.[109] In the NP-based sensor, the adsorption of the fluorescently labeled protein to the AuNPs leads to quenching of the fluorochrome. The preincubation of cells with AuNPs can reduce the subsequent adsorption of fluorescently labeled protein on NPs, thus increasing the fluorescence signal. The response is quite sensitive to less than five cells. This nanosensor is rapid, easy-to-use, high-throughput and independent of the cell viability, and therefore is a promising strategy for wide use in biochemical laboratories.

The AuNP-based quenching/recovery can be further designed as a sensor array for discrimination of normal and cancer cells through electrostatic conjugation of fluorescent polymer on AuNPs. The AuNP-quenched fluorescent polymer can be displaced by cells with concomitant restoration of fluorescence. This fluorescence response is dependent upon the level of displacement determined by the relative AuNP–polymer binding strength and cell–NP interactions. Through detection of the fluorescent pattern, a) different cell types; b) normal, cancerous and metastatic human breast cells; and c) isogenic normal, cancerous and metastatic murine epithelial cell lines can be rapidly and effectively distinguished.[110] A similar method has also been developed for bacterial sensing with a conjugated polymer (Sw-CO_2) featuring a branched oligo(ethylene glycol) side chain to suppress non-specific polymer–microorganism interactions (Fig. 2).[111] This AuNP-polymer sensor array-based strategy is different from current detection methods, which are based on specific recognition of intracellular/extracellular biomarkers (*e.g.*, DNA/RNA/proteins). It represents a new method for looking into diagnostic, biophysical, and surface science processes involving cell surfaces.[110,111]

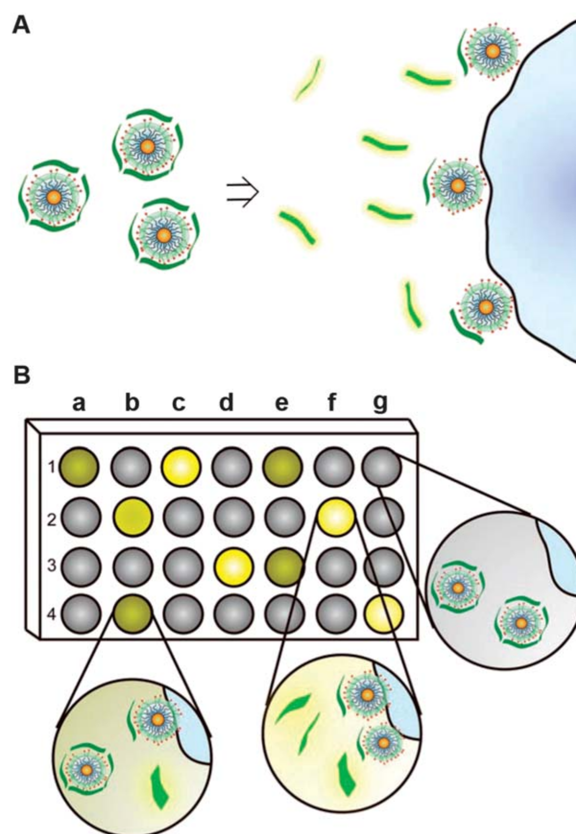

Fig. 2 Design of the NP-conjugated polymer sensor array. (A) Schematic representation of the displacement of anionic conjugated polymers from cationic NPs by negatively charged bacterial surfaces. (B) Schematic illustration of fluorescence pattern generation on a microplate. In the diagram, a–g on the microplate represent bacteria of different types, and codes 1–4 represent the polymer-NP constructs. (Adapted with permission from ref. 111. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.)

Luminescent Au NPs have also been exploited for cytosensing application, for example, *in vivo* pH monitoring and cell counting. pH is a key parameter for many biological processes and also an important indicator for disease progression.[43] Due to the Warburg effect,[112] the acidic pH is also characteristic of solid tumors (extracellular pH 6.0–7.0).[43] Generally, AuNPs do not exhibit pH-dependent interactions with live cells due to nonspecific serum protein adsorption.[113] To address this challenge, Zheng's group[44] took the advantages of a natural peptide, reduced glutathione, and the simplest stable aminothiol, cysteamine, as surface ligands to create luminescent negatively charged AuNPs. Due to the low affinity to serum proteins and the capability of minimizing nonspecific protein adsorption of glutathione and the electrostatic interaction between cationic cysteamine and cell surface, the obtained AuNPs exhibit not only high resistance to serum protein adsorption but also pH-dependent adsorption onto live cell membranes in a biological pH

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range from mildly acidic to neutral (5.3–7.4).[43] This research provided a example of employing surface chemistry to manipulate NP–cell membrane interactions in a native biological environment.

For cell counting, fluorescent water-soluble mannose-protected Au nanodots (Man-Au NDs) of 2.9 nm have been developed for facile detection of *Escherichia coli* (*E. coli*) using as little as 3 h.[114] Incubation of the nanodots with *E. coli* reveals that the Man-Au NDs bind to the bacteria, yielding brightly fluorescent cell clusters. The linear relationship between the fluorescence signal and the *E. coli* concentration is from 1.00×10^6 to 5.00×10^7 cells mL^{-1}, with the LOD of 7.20×10^5 cells mL^{-1}.[114]

3.1.1.4 Biofunctionalized luminescent silica NPs for cytosensing. Silica NPs are versatile platforms with many intrinsic features, such as low toxicity. These NPs are usually designed as multicomponent nanosystems, on which highly valued functions like light harvesting and signal amplification can be achieved.[7] Fluorescent silica NPs are one of the most widely developed and used NPs.[115–117]

The design of analyte-sensitive fluorescent probes based on silica NPs may, for example, offer important advantages compared to their molecular counterparts. The solubility and stability of the system in solution are conferred mainly by the silica structure; therefore, many compounds that cannot be used in an aqueous environment can instead be conveniently inserted, or covalently bound, to the silica matrix.[7] Because silica is transparent to visible light and is not involved in energy- and electron-transfer processes, the photochemical properties of dye doped silica NPs are mainly conferred by the doping species and, when present, by capping agents.[7]

Recognizing multiple cancer cells from complex living samples and accomplishing ultrasensitive and selective detection are critical issues for the early diagnosis of cancer cells. Silica NPs with flexible constitution and surface functionalization offer a powerful tool to address the issues. Silica NPs can be designed as a set of FRET silica NPs for multiplex cytosensing. To achieve this, different combinations of different dyes, chosen to allow an efficient-energy transfer between them, have been encapsulated in silica shells. This leads to different maximum emissions for the NPs, which can be obtained at a single wavelength excitation and used as the signatures of NPs. These FRET NPs are then conjugated with antibodies[118] or aptamers[58] specific for analyte cells, leading to sensitive and selective monitoring of multiple bacteria and cancer cells.

Different silica NPs with distinct functions can be used simultaneously for gaining better analytical performance. A two-silica-particle assay, for which silica-coated magnetic and fluorophore-doped silica NPs are both conjugated to aptamers, has been developed to detect and extract targeted cells in a variety of matrixes.[119,120] The MNPs permit the collection and enrichment of the cancer cells, whereas the fluorescent NPs clearly mark the cell for fluorescence detection. Through further using an innovative multiple aptamer approach, ultrasensitive detection can be achieved with the limit of detection down to 6.6 cancer cells.[59] The functionalization of a single-particle set with multi-aptamers leads to the recognition of potential variation possibilities from sample to sample. This approach allows for a screening assay empowered with the ability to characterize individual patient profiles which is the first step to achieving real personalized cancer therapies.

3.1.1.5 Biofunctionalized upconverting NPs for fluorescent cytosensing. High-quality upconverting NPs (UCNPs) linked to biological macromolecules are considered as novel fluorescent nanoprobes for investigation of biological processes. UCNPs, which emit in the visible range upon absorption of NIR photons, have attracted great attention as they benefit from a narrow particle size distribution, high photostability, narrow emission bandwidths, good cytocompatibility, and exhibit neither photoblinking nor photobleaching.[9,98,121] Moreover, by employing NIR excitation, one can suppress cellular autofluorescence, induce little photodamage to living cells, and achieve relatively deep penetration into tissues.[122,123] As a result, UCNPs have become one of the most promising NP systems for biological application.[124] Among the various kinds of upconverting NPs, $NaYF_4:Yb^{3+},Er^{3+}$ and $NaYF_4:Yb^{3+},Tm^{3+}$ NPs are known to be the most efficient systems.[121] The real-time tracking study with UCNPs ($NaYF_4:Yb^{3+},Er^{3+}$) coated by amphiphilic PEG-phospholipids at the single vesicle level in living cells has been achieved.[98] Inclusion or doping of Gd^{3+} ions in the host materials can endow UCNPs with an additional modality for magnetic resonance imaging (MRI).[121,125]

Recently, poly(ethyleneimine) capped $NaYF_4:Yb^{3+},Er^{3+}$ UCNPs have been exploited for cytosensing application. Making use of the temperature sensitive property of UCNP, a novel nanothermometer capable of accurately determining the temperature of solutions as well as in HeLa cancer cells has been developed.[124] The nanothermometer is based on the phenomenon that the intensity ratio between the green fluorescence bands of the Er^{3+} dopant ions ($^2H_{11/2} \rightarrow {}^4I_{15/2}$ and $^2S_{3/2} \rightarrow {}^4I_{15/2}$) changes with temperature. Further exploitation of unique properties of UCNPs and improvement in the optical upconversion efficiency and surface conjugation schemes will make UCNPs more versatile nanoparticle-based cytosensors for other cellular components, such as proteins and organelles, accelerating diagnostic and therapeutic applications thereof.[98]

3.1.2 Biofunctionalized AuNPs for SERS-based cytosensing. SERS has recently emerged as an alternative to fluorescence-based spectroscopy for intracellular NP sensors,[87,126] and is free from photobleaching and self-quenching of the marker molecule. The SERS effect is provided by the proximity of Raman active signature reporters to the surface of metal nanostructures,[127,128] which can be functionalized with molecular recognition motifs to render diagnostic tools for optical sensing and therapeutic studies.[48,129] The sensitivity of SERS measurements is one of the highest among analytical techniques, potentially leading to single molecule detection.[130] For SERS-based cytosensing, current research focuses on the following directions:

1) Developing novel gold nanostructure-based substrates. A class of biocompatible and nontoxic pegylated AuNPs coated with a protective layer of PEG has been developed by Nie's group[48] for *in vivo* tumor targeting and surface-enhanced Raman detection with large optical enhancements. When conjugated to single-chain variable fragment (ScFv) antibodies, the conjugated NPs are able to target tumor biomarkers such as epidermal

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growth factor (EGF) receptors on human cancer cells and in xenograft tumor models. The advantage of this NP platform is the facile conjugation of tumor targeting ligands to heterofunctional PEG linkers.

Various gold nanostructures have been reported for enhancement of Raman signals in intracellular chemical sensing, such as gold nanoshells,[131] nano-popcorn,[132] aperiodic arrays of AuNPs with different length scales,[133] and a glass capillary with a 100–500 nm tip coated with AuNPs.[134] Because SERS signals are known to be highly sensitive to the exact NP configuration, uncontrollable aggregation of NPs in cells poses difficulties. A solution to this problem is using NPs with fixed geometry as the SERS substrates, such as incorporating AuNPs on the outer surface pipette tip.[134] This nanopipette can be fabricated based on any standard equipment for cell microinjection.

2) Optimizing biofunctionalization format. The multivalent binding is usually considered to be essential for early-stage disease diagnostics.[132,135] Multifunctionalized gold nano-popcorn has been exploited for SERS assay for targeted sensing, nanotherapy treatment, and *in situ* monitoring of photothermal nanotherapy response during the therapy process (Fig. 3).[132] In the presence of LNCaP human prostate cancer cells, antibody and Ramam dye-attached aptamer co-conjugated nano-popcorn can undergo aggregation with cells and provide a significant enhancement of the Raman signal intensity by several orders of magnitude (2.5×10^9). As a result, it can recognize human prostate cancer cells at the 50-cells level in a 30 min assay time. By monitoring SERS intensity changes, one can monitor the photothermal nanotherapy response during the therapy process.[132]

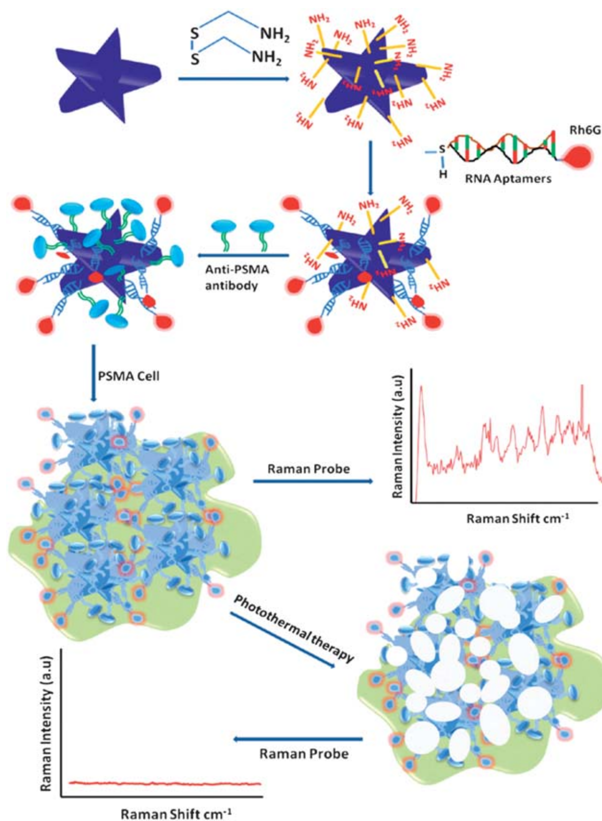

Fig. 3 Schematic representation of the synthesis of monoclonal anti-PSMA antibody- and A9 RNA aptamer-conjugated popcorn-shaped AuNPs. (Reproduced with permission from ref. 132. Copyright 2010 American Chemical Society.)

3) Designing highly sensitive Raman reporters. Commonly used Raman signature molecules include crystal violet, malachite green isothiocyanate, rhodamine-6G, Nile blue, 2-naphthalenethiol, TRITC (tetramethylrhodamine-5-isothiocyanate), DTTC and XRITC (Xrhodamine-5-(and-6)-isothiocyanate) *etc.*[32] A combinatorial approach has been developed to prepare sensitive near-infrared (NIR) SERS nanotags for cancer-cell detection and discrimination. In the study, after chemisorbing lipoic acid-containing tricarbocyanine compounds on AuNPs, the NPs are encapsulated by BSA using glutaraldehyde to form a cross-linked organic encapsulation layer around the gold and prevent the distortion of the reporter molecules. The obtained NPs are conjugated to HER2-recognition motifs for selective detection of cancer cells expressing HER2 receptors.[32]

3.1.3 Biofunctionalized NPs for CL cytosensing. The CL detection system is one of the most attractive analytical tools which possesses characteristics of rapidity, precision, low cost, and simple manipulation.[136,137] It has recently been introduced into *in situ* cytosensing,[138,139] which is often designed in a displacement assay format.[28,29] In principle, the specific binding of cell to aptamer can lead to release of a ssDNA sequence, which is the real analyte during assays. Because three components are involved in the assay, MNPs are used either to conjugate with aptamer, or link a sequence complementary to ssDNA for simplifying the separation procedure. Furthermore, the MNPs are also endowed with a signal amplification function.

For example, a displacement assay has been designed for aptamer-based detection of Ramos cells featuring a molecular beacon modified Fe_3O_4@AuNP for signal amplification and simplification of the separation procedure.[138] As seen from Fig. 4, the specific binding of target cells to the aptamer results in the release of a ssDNA sequence (3). The released ssDNA sequences (3) containing the NEase recognition site can hybridize with the molecular beacon (MB)-Fe_3O_4@Au conjugates without need for separation, and the resulting hybridization event activates the autonomous synthesis of the DNAzyme units in the presence of nicking endonuclease (NEase). The DNAzyme can

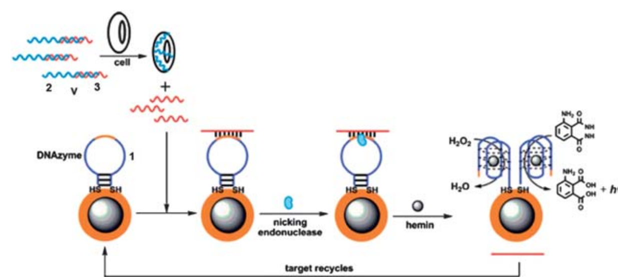

Fig. 4 Schematic representation of improved CL strategy for cancer cell detection based on the NEase assisted strand circular amplification process. (Adapted with permission from ref. 138. Copyright 2010 The Royal Society of Chemistry.)

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be used as catalytic labels for CL detection through catalyzing the oxidation of luminol by H_2O_2 to generate CL signals. The CL intensities increased with an increase of the target cell concentrations with a dynamic range from 100 to 10000 cells mL^{-1}. The limit of detection is 67 cells mL^{-1}.

In another CL sensing strategy, the magnetic NPs are co-modified with aptamer and RuNP-covered dendrimer-like DNA.[139] The latter is released after the displacement procedure, and the RuNPs can give a CL signal in the presence of luminal and H_2O_2 after dissolution in nitric acid. The CL intensity is proportional to the concentration of target cells ranging from 100 to 10000 cells mL^{-1} with a limit of detection of 62 cells mL^{-1}.

3.2 Biofunctionalized NPs for magnetic resonance cytosensing

Among a large variety of nanomaterials, MNPs have received considerable attention in the past decade because they offer precisely controlled size, ability to respond to noncontact manipulation, and enhancement of contrast in magnetic resonance imaging (MRI).[8] As a result, MNPs are increasingly important in many biomedical applications, including medical imaging, protein purification, bacteria capture, and drug delivery.[52,140] It also offers a simple and viable process for investigating cellular responses to mechanical perturbation of specific proteins *via* magnetic (*i.e.*, noncontact) modulation,[52] which would pave the way for establishing and regulating molecular pathways for a wide range of cellular functions.

In the meantime, MNPs with high magnetic moments and very small size are under active development for diagnostic magnetic resonance (DMR) sensing.[64] DMR sensing is based on the change of transverse relaxation time (ΔT_2) when detection targets in samples are recognized by MNPs.[141] The particles consisted of an elemental iron (Fe) core, an artificial ferrite shell ($Fe@MFe_2O_4$, M = Fe, Mn, Co) and a dimercaptosuccinic acid (DMSA) coating. For specific cancer cell detection, the MNPs are functionalized with HER2/neu antibodies. After incubation of cancer cells with MNP for 10 min, and removal of unbound MNPs, the T_2 values at different cell concentrations are measured and the cellular relaxivities, defined as the relaxation rate ($R_2 = 1/T_2$) per cell concentration, are obtained. The superiority of $Fe@MnFe_2O_4$ enables the detection of 10 cancer cells in the presence of abundant host cells (leukocytes) by DMR measurements. This result indicates highly magnetic NPs can be used for MR-based diagnostics. The particles can also benefit other applications, such as bio-separation (*e.g.*, proteins, cells) and the direct detection of biological targets using magnetometers.

MNP can also be combined with other type of NPs for assembly of hybrid NPs. The ability to selectively arrange nanosized domains of metallic, semiconducting, and magnetic materials into a single hybrid nanoparticle offers an intriguing route to modify nanoparticle properties, engineer nanomaterials with multiple functionalities or the enhanced properties of one domain.[142] For example, Ying and co-workers[143] synthesized Fe_2O_3/CdSe composite nanocrystals, and then coated a biofunctionalized silica shell on the nanocrystals to obtain biocompatible MNPs for labeling experiments of live cell membranes. The composite particles can be recovered using magnetic filtration. This combination of properties suggests the applicability for simultaneous imaging and magnetic-field directed movement of individual cells.

3.3 Biofunctionalized NPs for colorimetric cytosensing

Early and accurate detection of cancer is the key to an effective and ultimately successful treatment of cancer. Many current methods often rely on time-consuming techniques (*e.g.*, immunohistochemistry) or expensive advanced instrumentation (*e.g.*, flow cytometry).[144] The ability to rapidly identify low-abundance disease markers on the cell surface with instrument-free procedures is a tremendous challenge in disease diagnostics. Colorimetric methods, in particular, are extremely attractive for this purpose because they can be easily read out with the naked eye. There are two types of NP-based colorimetric cytosensing: 1) based on the intrinsic property of NPs to induce color change, and 2) relying on the color change of other molecules. In both of the two cases, the biofunctionalization of NPs endows the NPs with recognition ability.

For the first type, the unique physical properties, particularly localized surface plasmon resonance (LSPR), make AuNPs attractive building blocks for nanoscale signal transducers and/or signal amplifiers in a variety of colorimetric platforms. The AuNP-based colorimetric detection makes use of the color change, which arises from the interparticle plasmon coupling during AuNP aggregation and redispersion.[145] To regulate the assembly of AuNPs, biomolecules with recognition ability have been generally used to functionalize NPs, leading to colorimetric biosensors.[146]

A direct colorimetric assay for detection of diseased cells has been developed by Tan's group.[57] AuNPs modified with aptamers specific for CCRF-CEM cells are incubated with target cells (Fig. 5). The aptamer-directed assembly of AuNPs on the cell surfaces leads to a shift in the absorption spectra of the AuNPs, and the obtained larger μm-scaled gold structure exhibits significantly increased scattering and absorption coefficients. The assay is quite sensitive, since 1000 target cells can be readily detected by naked eye observation. In addition, different types of targets and control cells can be differentiated using this strategy. It can be expected that the aptamer-conjugated AuNPs can provide a economical and powerful tool for point-of-care diagnostics, and be expanded for large-scale screening of particular diseases.

In the case that single aptamer or antibody binding is not enough to achieve sensitive colorimetric detection,[58] design of stronger interactions between target cells and NPs is important, which can produce larger aggregates and lead to more significant changes of color. The combination of aptamers with antibodies to functionalize NPs, which affords multivalent binding, will provide a solution to increase the sensitivity and selectivity of colorimetric cytosensing. Ray's group[65] has conjugated oval-shaped AuNPs by multiple HER2 specific targets, anti-HER2 antibody and S6 RNA aptamer, to fabricate a nanoprobe for highly selective and sensitive detection of a breast cancer cell line. When multifunctional nanoprobes are mixed with breast cancer SKBR-3 cell line, a distinct color change occurs and he two-photon scattering intensity increases by about 13 times. Although remarkable progress has been made by design of this multifunctional nanoprobe, surface control of the nanoparticle

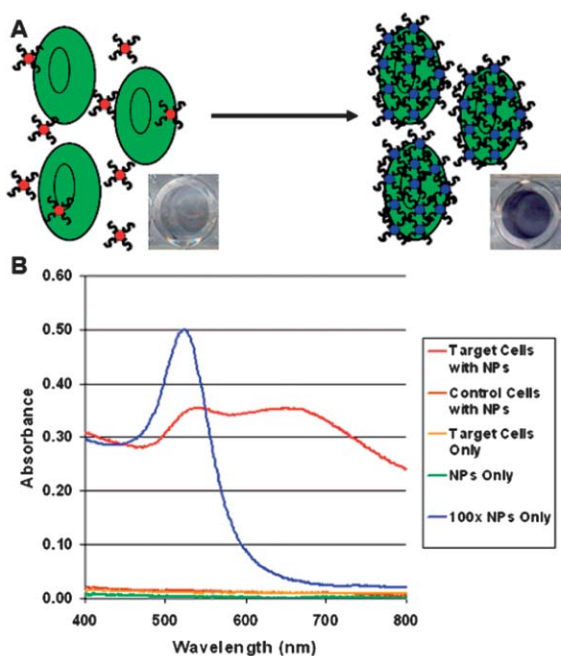

Fig. 5 (A) Schematic representation of the aptamer conjugated AuNP (ACAuNP) based colorimetric assay. (B) Plots depicting the absorption spectra obtained for various samples analyzed using ACGNPs. The spectra illustrate the differences in spectral characteristics observed after the ACAuNPs bind to the target cells. (Reproduced with permission from ref. 57. Copyright 2008 American Chemical Society.)

platform is still necessary to stabilize and maximize the assay response to fit the requirement of point-of-care diagnostics in complex environments.

For the second type, a colorful molecule is added to the analyte system, with the ability to display a color change in response to a recognition event. Besides fabrication of a fluorescent sensor array, Rotello's group[47] also developed a colorimetric sensor for rapid and sensitive detection of bacteria. In this approach, cationic AuNPs featuring quaternary amine headgroups are electrostatically bound to β-galactosidase, the activity of which is inhibited. Upon binding NP to bacteria, β-galactosidase is released, and the activity is restored, which can convert a pale-yellow substrate into a red product, providing a colorimetric readout. Compared with the fluorescent sensor array,[110,111] this colorimetric strategy involves an enzyme-base amplification, thus a much lower limit of detection (down to 10^2 bacteria mL^{-1}) can be obtained. An interesting attempt should be highlighted that the obtained color image in RGB mode was analyzed, and plots for individual channels were presented. The sensitivity for the green channel is higher than that of the other two channels. This result suggests a deeper investigation of data from colorimetric images for future study.

3.4 Biofunctionalized NPs for electrochemical cytosensing

Electrochemical techniques, with the remarkable advantages of low cost, user-friendly instrumentation, rapid detection, and adequate sensitivity and accuracy,[147–149] provide an ideal platform for point-of-care diagnosis.[144] Because of the unique properties of nanomaterials, there has been an explosion of interest in the use of nanomaterials for electrochemical cytosensing. Examples of nanomaterials include semiconductor QDs,[40,150] metallic NPs,[46,147,151] carbon nanotubes,[152–154] nanocomposites,[39,155] MNPs,[77] or nanostructured conductive polymers.[156] The significant roles of biofunctionalized nanomaterials in electrochemical cytosensing include design of the cell-compatible interface, facilitation of the electron transfer at the electrode interface, achievement of efficient transduction of the biorecognition event; enhancement of sensitivity and selectivity; and improvement of response times.[157] The electrochemical cytosensors can be divided into two types: one is using biofunctionalized NPs as the immobilization matrix of cells to fabricate a cytosensor, the other is employing the inherent property of NPs for enhancement of detection sensitivity.

3.4.1 Biofunctionalized NPs as immobilization matrix for electrochemical cytosensing.

A single living cell can be properly described as an electrochemical dynamic system. The voltammetric responses of the redox centers in living cells usually show irreversible electron transfer processes, which is related to the oxidation of guanine.[39,151,155,158,159] Anchoring cells on the electrode for producing electrochemical signals is a key step in the development of electrochemical cytosensors. However, conventional methods possess some limitations during the immobilization of cells on the electrode surface, such as instability, additional diffusional barrier, and decrease of cell viability.[149] Recent studies have focused on the design of biofunctionalized nanomaterials for both significant enhancement of the electrochemical response and keeping of cell viability for cell adhesion.[39,151,155,158,159]

Ju's group[39,147] developed different forms of biofunctionalized AuNPs to modify electrodes for electrochemical investigation of cell physiological function and cell surface biomarkers. The AuNPs attached on chitosan were used as a non-toxic biomimetic interface for immobilization of K562/ADM cells on the electrode surface, which provided an environment similar to a native system and allowed more freedom in orientation of the biomolecules, thus efficiently retaining the activity of living tumor cells and preventing cell leakage from the electrode interface.[147] Recently, gold nanocomposites, synthesized with polysaccharides as both the reducing and stabilizing agents, have been produced for cell immobilization.[39,149] The living K562 cells immobilized on an AuNP-chitosan nanocomposite gel exhibit an irreversible voltammetric response, which is used to monitor cell activity, and cellular adhesion and proliferation.[39] The major shortcoming of this kind of cytosensor is that the sensors are not specific to certain types of cells owing to the specificity-free nature of the biopolymers, thus they can not be used for cell discrimination. As a solution to solve this problem, biomolecules with recognition ability can be used to conjugate NPs for cell capture on electrodes. An antibody functionalized MNP has been fabricated by biotin-streptavidin interaction and used for specifically capturing cells and concentrating cells into a small volume at interdigitated array microelectrodes.[77]

Electrochemical cytosensors have been used for detection of cells based on the barrier function of cells by the electrochemical impedance spectroscopic (EIS) technique. Cells have excellent insulating properties and can affect the local ionic environment at the electrode/solution interface, which leads to a change of

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impedance.[160–162] The barrier depends on the biological status of the cells, including cellular viability and morphology, cell number, and adhesion, proliferation and apoptosis. During EIS measurements, the obtained electron transfer resistance (R_{ct}) reflects the blocking extent toward electron transfer of the modification layer on the electrode surface. EIS holds important advantages for sensitive and direct detection of cells in buffer solution, cell–cell interactions and cell–matrix interactions.[163] Theoretically, it can dynamically measure cellular movement at nanoscale with better resolution than conventional optical methods.[160]

In order to enhance the detection sensitivity of EIS-based cytosensors, lowering the electron transfer resistance of the coating on th electrode surface for cell capture and increasing the cytocompatibility of the coating are of main concern. Owing to the unique properties, biofunctionalized nanomaterials have been increasingly used for the fabrication of EIS-based cytosensors to improve cell immobilization, retain cell viability, increase electrode surface area and decrease the background impedance, thus enhancing detection sensitivity. AuNP-chitosan nanocomposite gel has been reported to be useful for impedance sensing of cells.[39] Using EIS, the binding of K562 leukemia cells can be monitored by the increasing R_{ct}, which correlates to the logarithm of the cell concentration. This sensor can be used to detect cells not only in suspensions but also on the electrode surface upon the proliferation of living tumor cells. For example, after a culture time of 120 h, drastically increasing resistance was observed, which was related to the apoptosis of cells. This electrochemical strategy is an effective and simple way for continuous online monitoring of cell proliferation and apoptosis when disposable electrodes are used.

The biofunctionalized NPs can be further integrated with other nanomaterials, such as single-walled carbon nanotubes (SWNTs) for enhanced detection sensitivity. By combination of the gelatin-stabilized AuNPs and carboxylic SWNTs, a novel nanocomposite has been assembled on the electrode to construct a nontoxic biomimetric interface for the immobilization of HL-60 cells.[149] More importantly, this work demonstrates that the carboxylic SWNTs and AuNP-gelatin may have a cooperative effect in facilitating the uptake of anticancer drugs into the targeted tumor cells, suggesting a potential application of the nanocomposites in cancer chemotherapy.

Researchers are currently attempting to exploit other electrochemical techniques in combination with biofunctionalized NPs to develop novel sensitive cytosensing mechanisms. Photoelectrochemistry, as a newly developed analytical method based on the photoelectrochemical properties of the materials, has promising potential in biological analysis.[164–166] Among semiconductor materials, CdS QDs, with the advantages of a narrow band gap and photoelectrochemical activity in the visible range, are widely used as photoactive materials. Functionalization of CdS QDs with biomolecules as recognition motifs and amine surface ligands as hole acceptors, can lead to the application of photoelectrochemical detection in cytosensing. Xu's group[167] has developed a photoelectrochemical cell sensor for the determination of SMMC-7721 human hepatoma carcinoma cells by using a photosensitive CdS polyamidoamine (G4) nanocomposite film (CdS-PAMAM). The film is further biofunctionalized by conjugating concanavalin A (Con A) for cell capture *via* glutaraldehyde bridges. The cell binding causes a decrease of photoelectrochemical current, which produces a cytosenser with a linear range of cell concentration from 5.0×10^3 to 1.0×10^7 cells mL^{-1} and detection limit of 5.0×10^3 cells mL^{-1}. This work displays a bright future for the photoelectrochemical method in cell detection.

Electrochemiluminescence (ECL) offers better performance than photoluminescence (PL) in many applications.[168] The possibility of avoiding excitation sources in fact leads to remarkably low noise, signal specificity, and easier design of the device. Electrochemiluminescence resonance energy transfer (ECL-RET), which is a powerful technique for probing changes in the distance between donors and acceptors,[150] is thus ideal for the sensitive detection of cells. For construction of the ECL-RET pair, antibody conjugated CdS QDs have been chosen as donor, with $Ru(bpy)_3^{2+}$ modified cells as acceptor. A novel ECL-RET protocol involving energy transfer from CdS QDs to cell-labeled $Ru(bpy)_3^{2+}$ has been used for sensitive detection of SMMC-7721 cells (Fig. 6).[150] The sensitivity is extremely high with the limit of detection down to 12.5 SMMC-7721 cells mL^{-1}, which attributes to the signal amplification of $Ru(bpy)_3^{2+}$ and the specific antibody–cell surface interactions. This technique avoids cell separation processes.

3.4.2 Biofunctionalized NPs as signal amplification labels for electrochemical cytosensing. The design of biofunctionalized NPs as nanoprobes with both specific recognition and signal amplification capabilities provides a powerful train of thought for the development of more sensitive electrochemical cytosensors.

Two kinds of signal amplification mechanisms are popular during the design of nanoprobes for electrochemical cytosensing: 1) based on the conjugated biomolecules, such as enzymes, and 2) relying on the intrinsic property of NPs. NPs offered a flexible platform for function design. Conjugation of NPs with both a recognition component and a signal amplification component has been widely used for construction of nanoprobes. A sensitive nanoprobe, prepared by co-conjugation of horseradish peroxidase (HRP) and Con A on AuNP, has been developed for cytosensing and cell surface carbohydrate assay.[46] The nanoprobe integrates the mannose recognition capability of Con A with enzymatic catalytic amplification. The high surface-to-volume ratio of AuNPs and the high molar ratio of enzyme to

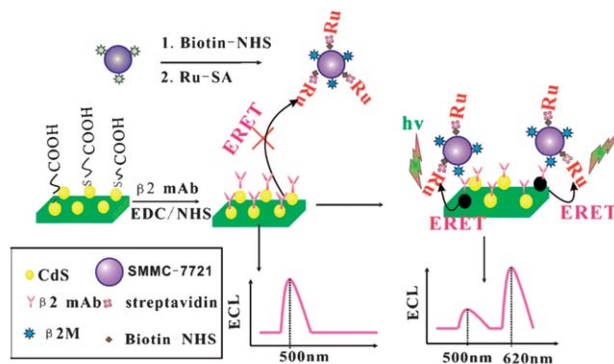

Fig. 6 ECL biosensor based on ECL-RET for determination of β2 M expressed cells. (Reproduced with permission from ref. 150. Copyright 2011 The Royal Society of Chemistry.)

Con A lead to a dual signal amplification for highly sensitive detection. The sensitivity is 34 times higher than that using simple HRP-Con A, verifying the advantages of using NPs for signal enhancement.

Different from the first kind, utilizing intrinsic properties of NPs can avoid the use of enzymatic substrates, thus simplifying the detection procedure. An AuNP-based electrocatalytic device for the specific detection and identification of tumor cells has been constructed on a screen-printed carbon electrode (SPCE) by Merkoçi's group.[144] AuNPs conjugated with antibodies can specifically bind cell surface proteins, and the electrochemical signal from catalytic properties of the AuNPs on hydrogen evolution allows quantification of the corresponding attached cancer cells. It can be envisaged that through the combination of a disposable SPCE array, this research paves the way for multiplexed screening of cells, and with the development of instrument miniaturization, this strategy might be suitable for point-of-care detection.

4. Biofunctionalized NPs for cell surface carbohydrate assay

Mammalian cells are covered with a dense and complex layer of carbohydrates, which may be the most abundant and diverse natural biopolymers.[169] These sugar chains are often covalently attached to underlying proteins or lipids, and form various structures. Carbohydrates play key roles in a myriad of biological events, including protein folding, trafficking, and stability, organ development, cellular adhesion, cell signaling, immune response, and pathological processes.[169,170] More importantly, aberrant expression of carbohydrates on a cell surface indicates certain disease states, such as inflammation and cancers.[171,172] In theory, carbohydrate can be used to diagnose, predict susceptibility to and monitor the progression of cancer.[172] With the aim toward improved understanding on the complex glycosylation machinery and the development of glycotherapeutics for major human diseases, great efforts have been made to develop sensitive and selective approaches for analysis of carbohydrates on cell surfaces.[173,174]

Mass spectrometry is the most widely used technique for decoding cell surface glycosylation. Although this technique can provide molecular details, it is time-consuming and not amenable to living cell interrogation due to the destructive sample preparation methods. As an alternative, recognition-based methods, which generally involve the use of lectins and antibodies with defined carbohydrate-binding specificities,[175] can offer nondestructive tools to profile cell surface glycans and correlate global changes in their expression with developmental stages and disease.[173]

Owing to the following advantages of biofunctionalized NPs, the combination of specific carbohydrate recognition methods with biofunctionalized NPs opens an avenue for *in situ* sensitive and selective analysis of cell surface carbohydrates. i) NPs are compatible with various glycan recognition elements, such as antibody, lectin or chemoselective molecules, thus providing versatile tools for carbohydrate analysis. ii) Multifunctionality of NPs is the key advantage of nanoplatforms over traditional approaches. Through regulating the surface and core properties of NPs or integrating different kinds of NPs, multiple functions, including recognition of carbohydrate motifs, signal amplification, separation and biosensing, can be readily achieved. Furthermore, the integration of multiple functions makes the detection more simple, which is beneficial for decreasing detection errors and improving accessibility to untrained users. iii) NPs can load multiple copies of carbohydrate recognition motifs to enhance the binding affinity, which possesses particular importance for the low-affinity carbohydrate-lectin interactions.[175] iv) The high loading of signal molecules on NPs can greatly improve the sensitivity of detection through dramatic signal amplification. v) The formation of NP-protein complexation can considerably stabilize the bound proteins against denaturation at the air/water interface,[6] which overcomes the unfavorable outcome of lectin denaturation for lectin-array based carbohydrate analysis methods.

Up to now, great progress has been made in the design of nanoprobes for recognition of (or competition with) cell surface carbohydrates, which leads to "direct" or "indirect" detection schemes, respectively. Current assay strategies can be divided into four main categories according to detection equipment: 1) fluorescence; 2) electrochemistry; 3) ECL and 4) scanometry.

4.1 Fluorescent assay of cell surface carbohydrates

QDs, with unique optical properties such as size-tunable emission wavelength, broad excitation profiles, and superior photostability, are the common choice for fabrication of nanoprobes used for fluorescent assay of cell surface carbohydrates.[16,176] One important advantage of QDs over molecular-structured dyes is their high brightness, which is at least ten times stronger than that of the best dyes. In addition, due to the photostability QDs can retain their luminescence when using UV light to photobleach the background autofluorescence of cells. Various types of lectins, which are carbohydrate binding proteins, have been employed to functionalize QDs for affording specific recognition functions. Compared with conventional fluorophores or fluorescent proteins, the larger size of QDs enables multiple loading of lectins, which is useful for increasing the binding affinity.

Recently, mercaptopropionic acid (MPA)-capped CdTe QDs have been used to attach Con A, a lectin with specificity for mannosyl groups, for the fabrication of mannose-specific nanoprobes.[177] The prepared QD-lectin nanoprobe can bind to cells in cell suspensions by specific recognition of Con A for cell surface mannosyl groups. After homogeneous incubation of cells with the nanoprobe solution and subsequent removal of the nanoprobe-bound cells, the decrease in fluorescence intensity of the nanoprobe solution is related to the cell number and the expression extent of the mannosyl groups on the K562 cells. This method can further be applied for the dynamic monitoring of the change of carbohydrate expression on cancer cells in the presence of a drug.

Flow cytometry is the dominant technique for cellular research and assay of cell surface carbohydrates. Fluorescein conjugated lectins are often chosen for the recognition process. The combination of a NP-based probe with flow cytometry provides a novel and versatile way for investigation of cell nature. Zhelev *et al.*[178] developed water-soluble COOH-functionalized CdSe QDs with plant-derived lectins for identification of leukemia cells from normal lymphocytes. The results demonstrated that QD-soybean

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agglutinin (QD-SBA) and QD-dolichos bifows agglutinin (QD-DBA) conjugates were appropriate fluorescent markers for identification of several leukemia cell lines.

Facile capture of target cells is beneficial for microscopic detection of cell surface carbohydrates. Thus the combination of MNPs with QDs can afford ways for both magnetic separation and fluorescence tracking. Single swelling poly(styrene/acrylamide) copolymer nanosphere has been used to encapsulate fluorescent QDs and γ-Fe_2O_3 MNPs, and simultaneously conjugated with lectin WGA for the fabrication of a novel type of trifunctional nanosphere.[179] The nanoprobe can be used for recognition of cancer cell surface-expressed sialic acid (SA) and *N*-acetylglucosamine. In a later work by the same group, different types of lectins, wheat germ agglutinin (WGA), peanut agglutinin (PNA), DBA were used to functionalize fluorescent-magnetic nanospheres.[180] The obtained nanoprobes can be used for qualitative and quantitative analysis of the glycoconjugates on the A549 cell surface. These lectin-modified trifunctional nanoprobes can not only quantify the different glycoconjugates on the A549 cell surface, but also recognize and isolate A549 cells. Therefore, these nanoprobes may be applied in mapping the glycoconjugates on cell surfaces and for recognition and isolation of targeted cells.

Although lectins are the most common carbohydrate recognition proteins used for fabrication of nanoprobes, they suffer from some limitations: relatively weak affinity of lectin–carbohydrate binding, cross binding with some types of lectins, and influence of chemical covalent process on the protein nature of lectins.[174] Thus, a novel type of recognition strategy has recently been developed based on the chemoselective recognition of carbohydrate by biocompatible small molecules.[181,182] For example, a new class of nanoprobe has been developed based on biofunctionalization of semiconductor QDs with small molecular phenylboronic acid (PBA) tags for highly specific and efficient labeling of SA on living PC12 cells (Fig. 7).[183] Among the diverse structures of glycans, SA with a nine-carbon backbone are commonly found at the terminal position of the sugar chains. The unique distribution and ubiquitous existence of SA on the cell membrane make them important mediators in various biological and pathological processes. These probes enable one-step labeling and continuous tracking of the cell surface sialic acid moieties without any pretreatment of living cells. The labeled sialic acids undergo quick internalization shortly after surface binding *via* endocytosis and eventually distribute in the perinuclear region.

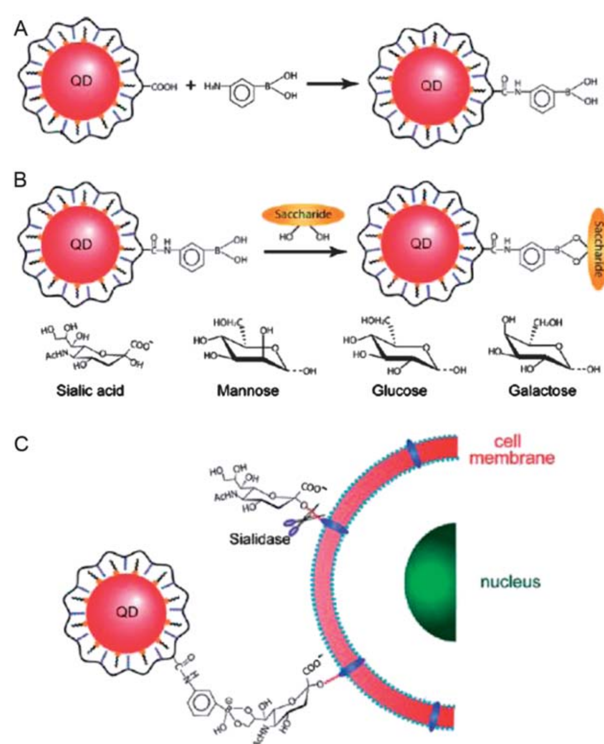

Fig. 7 Schematic illustration of QD conjugation and the use of QD probes for specific labeling of SA on living cells. (A) Conjugation of carboxylic acid functionalized QDs with 3-aminophenylboronic acid. (B) The reaction of PBA and diols in an aqueous solution. (C) Specific binding of QD with SA on living cells at the C-8,9 diol of SA. (Adapted with permission from ref. 183. Copyright 2011 American Chemical Society.)

4.2 Electrochemical cell surface carbohydrate assay

Biofunctionalized NPs have been employed as nanoprobes for electrochemical detection of cell surface carbohydrates with specific carbohydrate recognition ability or immobilization substrates for construction of electrochemical cytosensors.

4.2.1 Lectin conjugated nanoprobes. The development of electrochemical nanoprobes suitable for carbohydrate sensing, especially *in situ* cell surface carbohydrate monitoring, has emerged as an important area in bioassay. A nanoprobe prepared by co-conjugation of lectin and electroactive species thionine on AuNP has been developed for electrochemical study of the mannose and SA expression levels on normal and cancer cells.[184] In this work, cells were immobilized on electrode by lectin conjugated AuNP/multiwalled carbon nanotube (AuNP/MWNT) composite film. The nanoprobe for incubation with electrode-bound cells featured the amplification effect of AuNPs for the mass loading of thionine to achieve high sensitivity, and the recognition capability of lectin toward cell surface glycans. The thionine labels in the bioconjugates were recorded by electrochemical detection. The results indicated that mannose displayed high expression levels in both normal and cancer cells, while sialic acid exhibited enhanced expression in cancer cells as compared to normal ones.

In order to further increase the detection sensitivity, enzyme has been used to functionalize AuNPs to accomplish greater signal amplification owing to the catalytic ability of enzyme and large loading capability of NPs.[14] A dual-functionalized nanoprobe has been designed for highly sensitive and selective *in situ* evaluation of carbohydrates on living cells by integrating the specific carbohydrate recognition of Con A and enzyme-based catalytic signal amplification of HRP AuNPs.[46] The proposed method can be used for monitoring of dynamic variation of mannose expression on cancer cells in response to drugs, using swainsonine (SW), a mannosidase II inhibitor, as the model. Compared with other methods for monitoring dynamic glycan expression on cell surfaces, this strategy obviates the need for cell lysis, cell labeling and complicated equipment, and the

preparation of the nanoprobe does not involve covalent coupling, thus it can maintain the biological activity of proteins. The dual signal amplification coupled with enhanced electrical connectivity provides high sensitivity.

Although the sensitivity has been improved through functionalization of NPs with dual signal amplification, they suffer from the limitation that cells are in an immobilization format during the detection process, thus only part of the carbohydrate epitopes are exposed to the nanoprobe, and the overall expression information of carbohydrate on cells can not be obtained. To solve the problem, a "one molecule–two surfaces competition format" scheme, which allows *in situ* analysis of cell surface mannose moieties, has been developed (Fig. 8).[62] In this work, a lectin-functionalized QD probe is designed and a mannan carbohydrate monolayer is developed to compete with cell surface carbohydrates for recognizing the QD probe. The nanoprobe captured by the mannan monolayer can be then detected by an anodic stripping technique. This work does not require cell labeling, and overcomes the problems of active site inaccessibility and lectin denaturation, which are often encountered in the surface immobilization of proteins with high density. The anodic stripping of QDs is a powerful electroanalytical technique, because of its effective "built-in" preconcentration (deposition) step.[185] By combining the signal amplification from stripping analysis with the competition format, this method can reach the detection limit of 10^2 cells mL^{-1}, and the average Con A binding capacity of a single K562 cell has been estimated to correspond to 2.3×10^{10} mannose moieties. The QD-based electrochemical coding method can be further adapted for multiplexed cell surface carbohydrate assay through the usage of a series of QDs synthesized from distinct materials.

4.2.2 Biofunctionalized nanomaterials as immobilization substrates for electrochemical detection of cell surface carbohydrates. Nanomaterials have also been chosen as substrates for various functions. Their small size (1–100 nm) and correspondingly large surface-to-volume ratio can generate a roughened conductive-high-surface substrate interface after immobilization on the electrode surface, which can both favor the cell surface glycan recognition and enable the sensitive electrochemical and ECL detection. These strategies can be categorized into two main types: one is using nanomaterial-modified electrodes to immobilize cells and then detecting the signal from the recognition of lectin probe by voltammetry;[153,154] the other is immobilizing the lectin nanocomposite on electrodes, and then detecting the cell binding events by EIS.[186,187]

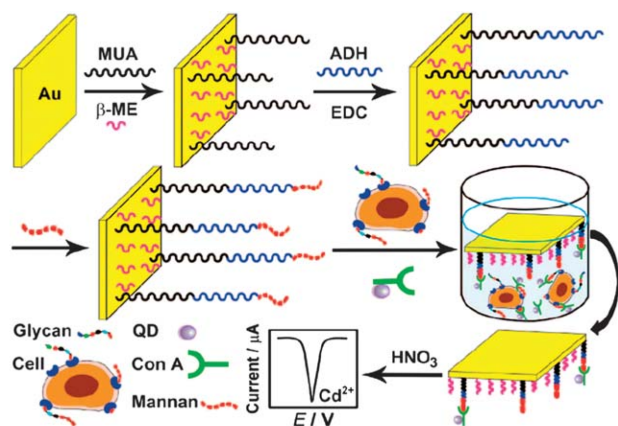

Fig. 8 Schematic representation of the monolayer fabrication and the competitive assay. (Reproduced with permission from ref. 62. Copyright 2008 American Chemical Society.)

The application of enzyme-linked lectin for specific recognition of carbohydrates on living cells captured on nanomaterial-modified electrodes provides enhanced sensitivity for evaluation of the expression extent of carbohydrate, owing to the signal amplification by the enzyme to produce abundant electroactive enzymatic product, increased electrode surface and accelerated electron transfer. Based on such a scheme, an electrochemical cytosensing strategy has been designed to obtain a glycan expression profile on K562[154] and BGC 823 cells.[153] Prior to recognition, the cells are immobilized on electrodes or disposable electrode array chips by arginine-glycine-aspartic acid-serine (RGDS)-functionalized SWNTs.[153,154] This strategy has further been used for effective monitoring of the dynamic variation of glycans on cancer cell surfaces during both drug inducement and erythroid differentiation of K562 cells.

Overproduction of certain glycoproteins is a common feature of tumors. For example, an energy-dependent transport protein named P-glycoprotein (P-gp), which is often overexpressed at tumor cells, is closely related to multidrug resistance phenomena.[188] Accordingly, the ability to characterize cell surface glycoprotein expression status is critical to advance chemotherapy of the malignant tumor. A strategy to detect P-gp on K562/ADM cells and quantify the cell number has been developed by Ju's group[147] using electrochemical immunoassay combined with the immobilization of cells on a highly hydrophilic interface. The interface is constructed by adsorption of AuNPs on a methoxysilyl-terminated (Mos) butyrylchitosan modified glassy carbon electrode (Au-CS/GCE) for cell capture. The incubation with P-gp monoclonal antibodies and then the secondary alkaline phosphatase (AP) conjugated antibody introduce AP to the cell surface. The bound AP leads to an amperometric response of 1-naphthyl phosphate, which is proportional to the logarithm of cell concentration in the range from 5.0×10^4 to 1.0×10^7 cells mL^{-1}. Later, some groups have expanded this strategy to investigate the expression extent of P-gp on HeLa cells[188] and BGC 823 cells.[189]

4.3 ECL cell surface carbohydrate assay

Owing to the low background noise and high sensitivity of the ECL technique, the combination of nanoprobes with the ECL technique has greatly contributed to *in situ* assay of cell surface carbohydrate expression. A facile ECL strategy for label-free monitoring of carbohydrate expression on living cells has been designed based on carbohydrate-functionalized CdS QDs/CNT nanocomposites, which act as ECL emitting species.[190] The ECL biosensor is fabricated by combining CNTs and mercaptopropionic acid-capped CdS QDs *via* a layer-by-layer method, and mannan is then coupled to QDs. The carbohydrate-functionalized CdS nanocomposites show high ECL sensitivity and good stability, and provide an effective 3D architecture for Con A recognition, which leads to a decrease of ECL intensity. When BGC cells are used for competition with the mannan-derivatized

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electrode to bind the Con A, the ECL intensity of the electrode increases. The increase magnitude depends on both the cell amount and the expression level of cell surface carbohydrates. The average amount of mannosyl groups on the cell surface has been obtained by this protocol. This method obviates cell and lectin labeling, thus the biological activities of cell and protein can be maintained to the largest extent.

Similar to electrochemical strategies, NPs have also been employed as immobilization substrates for fabrication of ECL biosensors for cell surface carbohydrate assay.[40] In this case, as shown in Fig. 9, the QDs with ECL property are functionalized with lectins and immobilized on the electrode. The specific capture of cells on lectin-QD modified electrodes can be monitored by ECL measurements. Through using various kinds of lectins (Con A, DBA, PNA and WGA) and comparison of cell-binding extents to different lectin-modified electrodes, the expression extent of different glycans on cell surfaces has been obtained, which suggests high expression of $(GlcNAc)_2$ and/or sialic acid, moderate expression of mannose, less Galβ1-3GalNAc and little GalNAc residues on the K562 cell surface. The results have been verified to be in good agreement with flow cytometric results.

4.4 Scanometric cell surface carbohydrate assay

AuNP-based colorimetric assay is now of particular interest due to the simple readout of signals from interparticle plasmon coupling. Unlike lectin-array-based fluorescent detection, the visual signal does not alter upon prolonged exposure to light, which allows direct diagnostic tests. Scanometric technique combines the advantage of colorimetric detection with the array format, thus has become an FDA-approved detection method and spurred the development of many related assays.[191] Here, the scanometric technology implies that the information-recording and acquirement procedure is performed with a flatbed scanner.[72] This assay utilizes the catalytic properties of the NPs in a subsequent amplification event to affect the reduction of Ag^+ in the presence of hydroquinone. The grayscale value of the resulting silver spots is then measured to quantify the amount of target.[192]

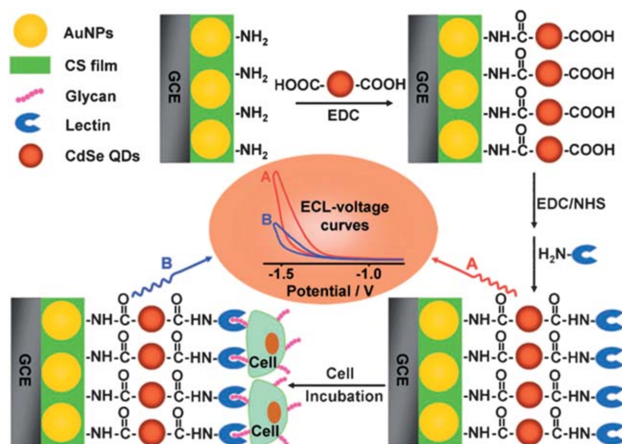

Fig. 9 Schematic representation of ECL cytosensor for monitoring cell surface carbohydrate expression. (Reproduced with permission from ref. 40. Copyright 2010 The Royal Society of Chemistry.)

Two strategies have been presented for scanometric assay of cell surface carbohydrates by using lectin to functionalize AuNP[63] and carbohydrate to functionalize AuNP.[72] In the first scheme, Con A modified AuNPs, which allow the competitive binding of cells and carbohydrate-modified slides, are used to reflect the carbohydrate expression extent on the cell surface by scanometric assay.[63] Compared with other existing methods, this strategy allows the cells to be interrogated at their original status, without the need to peel cells off a glassy substrate. In addition, by the usage of glass slides, high-throughput detection can be achieved. However, this method can not provide accurate amounts of certain types of carbohydrates on cell surfaces. The second scheme is designed by integrating the bioconjugation and aggregation of glyconanoparticles, silver signal amplification, and spot test.[72] As shown in Fig. 10, in the presence of lectin, the glyconanoparticles exhibit fast aggregation. The aggregation process can be inhibited by the specific recognition of lectin by the carbohydrate on the cell surface. The average number of cell surface mannose motifs can be conveniently read out. This largely noninstrumental method takes the advantages of a NP-based recognition and an aggregation regulated signal amplification, and avoids cell pretreatment and labeling processes. It possesses potential applications in clinical diagnosis and elucidation of carbohydrate functions on living cells.

4.5 Limitations of NP-based cell surface carbohydrate assays

Although the unique properties of NPs have offered many advantages for their applications in this field, they still have some limitations:

i) The cell surface glycans exist in diverse structural configurations. For cells in the disease state, simultaneous detection of the alteration of various kinds of glycans is essential for clinical dianostics, which needs the preparation of many types of nanoprobes with distinct signals. This may be hard to achieve for nanoprobe-based detection schemes, compared with lectin array-based assay. A possible solution is the use of a NP-based differential sensor array approach.[111]

ii) NPs must be amenable to cellular applications, including cell-compatibility, solubility, limited non-specific binding and long-term stability in buffered saline solutions under physiological pH. The complex architecture of nanoprobes has long posed considerable technical hurdles, compared with using conventional fluorophore- or enzyme-labeled lectins.

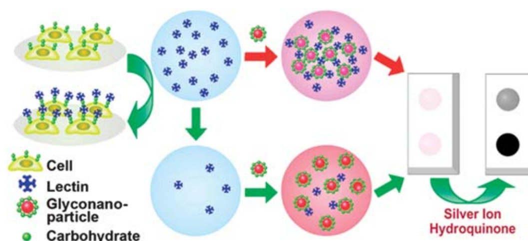

Fig. 10 Scheme of the scanometric strategy for *in situ* detection of mannose groups on living cells. (Reproduced with permission from ref. 72. Copyright 2010 American Chemical Society.)

iii) Compared with small dyes, the surface coating of NPs will lead to a larger hydrodynamic diameter of NPs, which causes steric hindrance for recognition of small carbohydrate motifs on cell surfaces, thus placing restrictions on studying spatially confined or crowded regions of the cells.

iv) The polyvalency of nanoprobes is a double-edged sword for cell surface carbohydrate assay. Although it can increase the binding affinity, it may also induce the cross-linking of target carbohydrate molecules on cell surfaces and activate signaling cascades, thus perturbing the behavior of the cells.

5. Summary and outlook

NPs present a versatile synthetic scaffold for the creation of detection systems for analyzing species in/at living cells. Biofunctionalized NPs provide a suitable platform for simplifying system design through the incorporation of the recognition of targets and the transduction of binding events. In this Feature Article, we have comprehensively reviewed the current research regarding the use of biofunctionalized NPs in cytosensing and cell surface carbohydrate assay. Various conjugation strategies, functionalized nanomaterials and related techniques have been discussed.

Further development of biofunctionalized NPs in cytosensing will lie in 1) the exploitation of novel nanoprobes for both recognition and signal transduction, 2) the employment of recognition systems with higher affinity for biofunctionalization, 3) the combination with equipment for surface biofunctionalization, 4) careful design of cell-involved experimental setup. Although various types of NPs have currently been used in cytosensing, developing novel nanoplatforms will be of continuous interest in biological applications. This can be addressed in two parallel fashions: exploiting novel nanomaterials, such as man-made nanomachines,[193] particularly autonomously moving synthetic nanomotors, which afford the pickup and transport capabilities for diverse payloads; and trying other combinations of nanomaterials, which may open the door to new and important cytosensing applications.[142] Surface functionalization of NPs is undoubtedly the most essential and fundamental factor. New conjugation strategies, such as barnase and barstar noncovalent binding system or click chemistry-based covalent linker *etc.*, will greatly accelerate the development of this field. The functionalization of NPs can also be achieved through combination with other equipment, for example capillary microfluidic devices to endow NPs with more flexible assembly format and customized functions.[194] These create new opportunities for expanding NPs to wider cytosensing applications, including high-throughput or multiplex bioassays. The interaction between NPs with cells usually relies on the size, shape and surface coating of NPs. Recent research has reflected that the sedimentation and diffusion of NPs also influence the interaction.[195] Thus careful choice of experimental setups is essential, for example, upright or inverted cell culture configurations.

Although tremendous success has been made, the concern of long term toxicity of nanomaterials is still an important issue to be addressed. Despite a significant surge in the number of investigations on the cytotoxicity of some kinds of NPs, QDs, AuNPs *etc.*, the knowledge about the cytological and physiological mediators of these effects is currently limited.[85] Further understanding of the cytotoxicity of nanoparticles will help to offer a better and more efficient way to apply nanoparticles in various biomedical applications.[195]

Owing to the particularly exciting application of biofunctionalized NPs for ultrasensitive and selective detection of cancer cells, it can be considered as a potential revolution in disease diagnostics, especially in cancer diagnosis and management. The integration of nanotechnology with chemical biology, organic chemistry and molecular biology will produce multifunctional nanoprobes for both sensitive diagnostics and delivery of drugs without systemic toxicity, which brings unprecedented opportunities for the future of cancer diagnosis and therapy.

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