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NANOPARTICLES AND THEIR APPLICATIONS IN CELL AND MOLECULAR BIOLOGY

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Abstract

Nanoparticles can be engineered with distinctive compositions, sizes, shapes, and surface chemistries to enable novel techniques in a wide range of biological applications. The unique properties of nanoparticles and their behavior in biological milieu also enable exciting and integrative approaches to studying fundamental biological questions. This review will provide an overview of various types of nanoparticles and concepts of targeting nanoparticles. We will also discuss the advantages and recent applications of using nanoparticles as tools for drug delivery, imaging, sensing, and for the understanding of basic biological processes.

1. INTRODUCTION

Nanotechnology is a relatively new branch of science that has found a wide range of applications that range from energy production to industrial production processes to biomedical applications.[1] One of the key applications is in biology and biomedical research. Nanoparticles (NPs) can be engineered to possess unique composition and functionalities, which can provide novel tools and techniques that have not previously existed in biomedical research. For example, NPs can be used to image biological processes on the cellular level. They can also be utilized to detect analytes at the attomolar range. In this review, we aim to discuss the types of NPs and their potential application in biology and biomedical research.

2. TYPES OF NANOPARTICLES

There are many types of NP platforms with differing size, shape, compositions, and functionalities. Furthermore, each type of NPs can potentially be fabricated using different techniques, such as both nanoprecipitation and lithography for polymeric NPs. While it is not within this manuscript's scope to discuss the differences in NP platforms and their fabrication in detail, we will discuss the major characteristics and functionalities of each NP that are relevant for biomedical research.

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Liposomes

The first NP platform was the liposomes. Liposomes were first described in 1965 as a model of cellular membranes.[2] Since then, liposomes have moved from a model in biophysical research to one of the first NP platforms to be applied for gene and drug delivery. Liposomes are spherical vesicles that contain a single or multiple bilayered structure of lipids that self-assemble in aqueous systems.[3] Unique advantages imparted by liposomes are their diverse range of compositions, abilities to carry and protect many types of biomolecules, as well as their biocompatibility and biodegradability.[3–4] These advantages have led to the well-characterized and wide use of liposomes as transfection agents of genetic material into cells (lipofection) in biology research.[5] Lipofection generally uses a cationic lipid to form an aggregate with the anionic genetic material. Another major application of liposomes is their use as therapeutic carriers since their design can allow for entrapment of hydrophilic compounds within the core and hydrophobic drugs in the lipid bilayer itself.[6] To enhance their circulation half-life and stability *in vivo*, liposomes have been conjugated with biocompatible polymers such as polyethylene glycol (PEG).[3] Liposomes can also be functionalized with targeting ligands to increase the accumulation of diagnostic and therapeutic agents within desired cells. Today, there are twelve clinically approved liposome-based therapeutic drugs.

Albumin-bound

Albumin-bound NPs (nab) uses the endogenous albumin pathways to carry hydrophobic molecules in the bloodstream.[7] Albumin naturally binds to the hydrophobic molecules with non-covalent reversible binding, avoiding solvent-based toxicities for therapeutics.[8] As a result, this platform has been successfully adapted as drug delivery vehicle. Abraxane, a 130-nm nab paclitaxel was approved by the FDA in 2005 for the treatment of metastatic breast cancer.[9] Abraxane concentrates in cells through albumin receptor (gp60)-mediated transport in endothelial cells. It may also target the albumin-binding protein SPARC (secreted protein acidic and rich in cysteine), which is overexpressed in certain tumors.[10] Further understanding of the mechanism of action may lead to better targeting and development of novel therapeutics using the nab platform.

Polymeric

Polymeric NPs formed from biocompatible and biodegradable polymers have been extensively investigated as therapeutic carriers.[11] Polymeric NPs are formulated through block-copolymers of different hydrophobicity.[12] These copolymers spontaneously assemble into a core-shell micelle formation in an aqueous environment.[13] Polymeric NPs have been formulated to encapsulate hydrophilic and/or hydrophobic small drug molecules, as well proteins and nucleic acid macromolecules.[14] The NP design can allow for slow and controlled release of drug at target sites. Polymeric NPs are usually able to improve the safety and efficacy of the drugs they carry. Functionalizing polymeric NPs with targeting ligands for improved drug delivery has been an important area of investigation since polymeric NPs are unique in their ability to be tailored prior to particle assembly. The incorporation of targeting ligands on the NPs can lead to their increased uptake along with their cargo, leading to enhanced therapeutic outcomes.

Another type of polymeric NP is dendrimers. Dendrimers are regularly branched macromolecules made from synthetic or natural elements including amino acids, sugars, and nucleotides.[15] They have a central core, interior layers of branches, and an exterior surface.[16] The varied combination of these components can yield dendrimers of well-defined size, shape, and branching length/density.[17] As a result of their unique design, dendrimers can be developed as sensors as well as drug and gene delivery carriers. Dendrimers can be loaded with small molecules in the cavities of the cores through chemical linkage, hydrogen bond, and or hydrophobic interaction.[18] The exterior surface can also be readily modified to produce chemical functional groups for molecular targeting groups, detecting and imaging agents, and therapeutic attachment sites.[19]

Iron oxide

Iron oxide NPs are widely studied as a passive and active targeting imaging agent as they are mainly superparamagnetic. The superparamagnetic iron oxide NP (SPION) generally have an iron oxide core with a hydrophilic coat of dextran or other biocompatible compound to increase their stability.[2021] The most widely used SPIONs consist of a magnetite (Fe_3O_4) and/or maghemite (γFe_2O_3) core. These NPs exhibit size-dependent superparamagnetism, which allows them to become magnetized with the application of an external magnetic field and exhibit zero net magnetization upon removal of the magnetic field. SPIONs have been successfully used as T2-weighted magnetic resonance (MR) contrast agents to track and monitor cells.[22] SPIONs have several advantages over conventional gadolinium-chelate contrast agents including decreased toxicity and increased imaging sensitivity and specificity.[23] SPIONS can also be degraded to iron and iron oxide molecules that are metabolized, stored in cells as ferritin, and incorporated into hemoglobin.[23] Currently, two SPIO agents, ferumoxides (120–180 nm) and ferucarbotran (60 nm) are clinically approved for MRI. SPIONs have also been used in molecular imaging applications such as the detection of apoptosis[24] and gene expression.[25] SPIONs can be functionalized with magnetic, optical, radionuclide and specific targeting ligands for multimodal imaging. They can also potentially be used as non-invasive diagnostic tools and as drug delivery vehicles.[26]

Quantum dot

First discovered in 1980, quantum dots (QDs) are semiconductor particles that are less than 10 nm in diameter. QDs display unique size-dependent electronic and optical properties.[27] Most QDs studied consist of a cadmium selenide (CdSe) core and a zinc selenide (ZnS) cap.[28] The absorption spectra of these particles are very broad and emission is confined to a narrow band. QDs can also emit bright colors, have long lifetimes, high efficiencies and are stable against photobleaching. They can be generated to have different biochemical specificities and can be simultaneously excited and detected. As a result, QDs have several significant advantages over many organic fluorophore dyes for optical applications. They are widely used in biological research as fluorescence imaging tools for applications such as cell labeling and biomolecule tracking.[293028] The small size of quantum dots also enables them to be suitable for biomedical applications such as medical imaging and diagnostics.[31]

Gold

Gold NPs offer many size-and-shape dependent optical and chemical properties, biocompatibility, and facile surface modification.[21] Gold NPs can strongly enhance optical processes such as light absorption, scattering, fluorescence, and surface-enhanced Raman scattering (SERS) due to the unique interaction of the free electrons in the NP with light.[32] These properties have enabled the realization of gold NPs in many applications such as biochemical sensing and detection, biological imaging, diagnostics, and therapeutic applications. Sensing techniques include the use of gold NPs in colorimetric arrays and the use of gold NPs as substrates in SERS to significantly enhance Raman scattering, allowing for spectroscopic detection and identification of proteins and single molecules at the NP surface.[33] Gold NP probes have also been used to detect heart disease and cancer biomarkers.[3435] They can also transform absorbed light into heat and therefore, have high potential for infrared phototherapy.[36]

3. TARGETED NANOPARTICLES

The concept of targeting has become a significant focus in biological research in recent years. In proteomics, targeted proteomics has gained traction as an approach to address specific biological questions by focusing on a subset of proteins of interest.[37] In genetics, gene targeting in mice has become a gold standard for determining gene function in mammals.[38] In the areas of drug discovery and molecular therapeutics, the development of small molecules and monoclonal antibodies such as imatinib[39], trastuzumab[40], bevacizumab[41], and rituximab[42] have considerably changed the treatment of cancer.

NPs show much promise in biological applications. In particular, NPs offer many unique properties that enhance or confer advantages over current techniques in biological and biomedical research. As a result, there has been significant interest in applying targeting concepts to NP design. NPs can be targeted by active targeting and passive targeting under *in vivo* conditions.

3.1 Passive Targeting

In the case of passive targeting, NP systems have been successfully developed for cancer therapy by taking advantage of tumor tissue biology. Normal tissue vascular biology has an organized structure while tumor vasculature is irregularly branched and disorganized.[43] Tumors also have high vascular density, increased vascular permeability, and impaired lymphatic drainage, an attribute of solid tumors and inflamed tissue.[43–44] Together, these features are known as the enhanced permeability and retention (EPR) effect, which allows NPs to accumulate preferentially in tumor tissue.[44] NPs have extended retention times in tumor tissue, which results in higher concentrations than in other tissues. Properties that mediate this passive targeting process include particle composition, size, shape, and surface characteristics.[45] Thus, NPs can be engineered to better target a particular tissue or cell by optimizing their physicochemical characteristics.

3.2 Active Targeting

Active targeting involves the use of targeting ligands for enhanced delivery of NP systems to a specific site. Typical targeting ligands include small molecules, peptides, antibodies and their fragments, and nucleic acids such as aptamers.[14] These ligands have all been conjugated to NPs.[14]

Conjugating targeting ligands to NP surfaces can be performed *via* covalent and non-covalent methods. With covalent conjugation, the same chemical methods for functionalization can be applied to various types of NPs since conjugation of functional groups to the NP surface is dependent on the functional groups on the NP surface and the functional groups of the ligand being conjugated. Certain conjugation techniques are also suitable for specific targeting ligand classes. The maleimide-thiol coupling is commonly used for conjugation of peptides, antibodies and their fragments to NPs. In this reaction, maleimides (maleic acid imides) spontaneously reacts with sulfhydryl groups at pH 6.5 to 7.5. Thus, nanoparticle surfaces incorporated with maleimide modified polymers or lipids can be readily conjugated with targeting ligands engineered with thiol groups or cysteine, which has a thiol side chain. Another commonly used conjugation method is the carbodiimide-mediated amide coupling between carboxyls and amines to form amide bonds. In this reaction, carboxylate groups on a molecule can be converted to active esters by using a variety of carbodiimides such as the water-soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxy-succinimide (NHS) or sulfo-NHS. The activated ester intermediate can react with a primary amine to form an amide bond. This method is generally preferred for aptamers, which can be modified with amines, and small molecules, which can contain carboxylates or primary amines. Click chemistry reactions are another method used to conjugate functional groups with high yield and selectivity under moderate reaction conditions. One of the most popular reactions with this class is the [3+2] cycloaddition between alkynes and azides.[46]

Conjugating targeting ligands to the NP surface can facilitate active targeting of NPs to receptors that are present on target cells, leading to enhanced cell internalization and/or specific uptake through receptor-mediated endocytosis.[47] For NP delivery of therapeutics, these advantages result in higher drug concentrations and reduced systemic toxicities compared to non-targeted NPs and their small molecule counterparts. Thus, there has been intense interest in identifying new disease biomarkers and their ligands for use in targeted drug delivery. Targeted NPs can also identify molecular targets with good affinity and selectivity. These NPs can bind to analytes, pathogens, and biomarkers, amplifying their signal for detection and molecular imaging.

4. NANOPARTICLE APPLICATIONS IN BIOLOGY

4.1 Nanoparticles as sensors

NPs have been employed in sensors for a variety of applications including detecting analytes at very low concentrations, detecting and separating pathogens, detecting and capturing cells, and detecting molecular and cellular functions.

4.1.1 Nanoparticles for analyte detection—The development of new sensing techniques for biological analytes such as DNA, RNA, and proteins are leading to more sensitive and efficient detection of these analytes at low concentrations. Many of these new techniques have taken advantage of the unique properties of NPs. NPs have large surface area to mass ratio, small size, and composition dependent properties that can enable the use of surface ligands as a way to amplify the detection threshold or provide more rapid detection.[48] The ability to easily functionalize NPs with targeting ligands can also enable specificity in binding and signaling of the analytes, allowing for more efficient detection. Development of NP biosensors has primarily focused on the use of inorganic NPs, particularly metallic or magnetic NPs.

NP platforms such as gold NPs have been extensively used as sensors due to their surface chemistry. One method of using the unique surface chemistry of gold NPs for signal transduction amplification is the bio-barcode method developed by the Mirkin group. This method has been applied for protein[49] and DNA[50] target detection. For target DNA detection, PCR-like sensitivity can be achieved. This technique has been reported for the detection of cytokines at a 30 aM (attomolar) concentration range[51] and for a soluble pathogenic biomarker for Alzheimer's disease at a concentration around 100 aM.[52] More recently, the group used this method to detect prostate-specific antigen (PSA), a commonly investigated cancer biomarker that can indicate the presence of prostate cancer when expressed at elevated levels.[53] In the bio-barcode assay approach, DNA-functionalized gold NPs (30 nm) are conjugated to PSA-specific antibodies to generate PSA gold NP probes (Figure 2).[5455] The DNA strands are the bio barcodes. A magnetic microparticle (MMP) functionalized with monoclonal antibodies to PSA are mixed with the PSA target protein. After a washing step, the PSA gold NP probes are added to the MMP-bound PSA. After further separation by a magnetic field application and wash steps, the PSA-specific DNA barcodes are released into solution and analyzed using the scanometric assay, which takes advantage of gold NP catalyzed silver enhancement. This assay achieved a sensitivity of 330 fg/mL of PSA.

The DNA bio-barcode method combines the ability to conjugate a large number of DNA strands onto gold NP surfaces and the detection of these DNAs using techniques such as PCR amplification or scanometric assays. Another approach for sensing that uses optical properties of the NP surface has been developed by the Rotello group.[56] They found that anionic fluorescent polymers may reversibly bind and dissociate from functionalized cationic gold NPs through non-covalent chemical interactions. When the fluorescent polymer is bound to the NP, its fluorescence is quenched. When it is dissociated from the NP, the fluorescence is restored. This phenomenon is the basis of a “chemical nose” system.[57] In this system, the target protein is detected according to their different fluorescence responses upon binding to different types of surface-modified gold NPs. A fluorescence response pattern is generated and then analyzed through statistical methods. This process can detect, quantify, and distinguish different molecular targets from each other. This process was initially developed for the detection and sensing of proteins,[57] but has also been adapted for the detection and sensing of bacteria,[58] for the detection of physicochemical differences between healthy, cancerous and metastatic human breast cells,

and for the differentiation of isogenic healthy and transformed cells (Figure 3).[5960] "Chemical nose" sensing also provides the advantage of not using antibodies for detection since the identity of the target analyte does not need to be known in order to detect the analyte.

Gold NPs can also enhance surface-enhanced Raman scattering (SERS) for detection of and identification of biomolecules at the NP surface. The technique SERS has been frequently used to detect specific analytes through their unique vibrational spectra. The narrow width of SERS spectra allows for multiple analyte detection within complex mixtures, including detection down to the single-molecule level.[33] Thus, SERS have been used for ultrasensitive detection of biomolecules such as glucose,[61] hemoglobin,[62] bacteria,[63] and viruses.[64] Using this approach, a group demonstrated that gold NPs encoded with Raman reporters and conjugated with single-chain variant fragment (ScFv) antibody can target cancer biomarkers such as epidermal growth factor receptors (EGFR) *in vitro* and *in vivo* (Figure 4).[6566] Recently, investigators also demonstrated a SERS system based on a thin-film NP array self-assembled at the liquid/liquid interphase for multi-phase trace analyte detection.[67] The group was able to detect single phase analytes as well multiple analytes dissolved in water and organic phases.

Nanosensors have also been developed that exploit the magnetic properties of magnetic NPs such as SPIONs. There is considerable interest in magnetic NPs since they have strong magnetic properties and most biological samples exhibit negligible magnetic susceptibility.[68] SPIONs have been used as magnetic tags for many different types of sensors including giant magnetoresistive (GMR) biosensors, which are based on the binding of magnetic particles to a sensor surface.[69] The magnetic fields of the particles can alter the magnetic fields of the sensor, resulting in changes in the electrical resistance of the sensor. Recently, Wang and colleagues at Stanford University have demonstrated the use of this technique for a protein detection assay in which an array of GMR sensors is used to detect binding events of proteins to arrays of surface-bound antibodies with the use of SPIONS as magnetic tags.[70] In this assay, the target antigen is between two antibodies, one bound to the sensor and the other tagged with a SPION. The presence or absence of the magnetized NP is detected by the underlying sensor. The group demonstrated the assay to be matrix insensitive to various biological fluids, but still capable of detecting proteins down to attomolar concentrations and over an extensive range of concentrations. Using a similar strategy, they have also demonstrated the detection of cancer-associated proteins in 50% serum at sub picomolar concentrations using MACS particles (commercialized SPIONs for cell separation).[71] Recently, they have used streptavidin functionalized antiferromagnetic NPs to detect DNA with high sensitivity (10 pM).[72]

The superparamagnetic property of IONPs is also the basis of magnetic relaxation switching. The Weissleder group has studied magnetic relaxation switches consisting of 3–5 nm SPIONs coated with 10 nm thick dextran and stabilized by crosslinking.[73] Targeted SPIONs are developed by functionalizing them with amino groups for the attachment of a range of sulfhydryl-bearing molecules.[74] The nanoswitches can undergo reversible assembly (clustering and declustering) in the presence of a molecule that is recognized by the ligands immobilized on the NP, resulting in a change in the transverse magnetic relativity ($1/T_2$) of

surrounding water protons. The changes in relaxation rates as a result of NP assembly can be used to detect a variety of biological targets including DNA and proteins at the low femtomole level (0.5–10 fmol).[75] Recently, the Weissleder group developed a portable chip-based diagnostic magnetic resonance (DMR) system for rapid and quantitative detection of biological targets.[76] The DMR also uses IONPs as sensors to amplify molecular interactions caused by assemblies of SPIONs. They demonstrated proof of concept by detecting with high sensitivity the presence of proteins in parallel and by detecting bacteria. Compared to the benchtop NMR relaxometer, the DMR system showed a mass detection limit improved by two orders of magnitude (detection limit of 15 fmol).

Magnetic NP relaxation sensors have also been used in which the basis of the assay is the relaxation of the magnetic moments within magnetic NPs.[77] Brownian relaxation is the dominant mechanism for these NP biosensors. In this method, NPs form aggregates in recognition of target analytes, leading to a larger hydrodynamic size and thus slower Brownian relaxation responses than individual NPs. Using this principle, a group recently detected a bacterial antibody at the sensitivity of 0.3 nM with an AC susceptometer[78].

4.1.2 Nanoparticles for pathogen detection and separation—Various NP platforms have been explored as sensors for detection and separation of pathogens. Most strategies for pathogen detection have utilized optical and magnetic properties of NP platforms. One of the most common methods used for the detection of bacteria has been through the use of magnetic biosensors that involves direct immunological reactions using magnetic NPs coated with antibodies against surface antigens.[7980] One group applied this immunomagnetic approach in a novel microfluidic device to attract molecules bound to magnetic NPs from one laminar flow path to another *via* a local magnetic field gradient.[80] Using this device, E. coli labeled with biotinylated anti-E.coli antibody and bound to streptavidin-coated SPIONs were efficiently separated from solutions containing densities of red blood cells similar to blood.

The Weissleder group has also used antibody-coated NPs in a microfluidic device to detect bacteria.[81] They also reported that core/shell NPs with Fe metal cores have enhanced sensitivity compared to IONPs for the detection of bacterial cells. Recently, the group also developed an assay based on a magnetic barcoding strategy that did not require antibodies and could detect single-gene mutations.[82] In this approach, PCR-amplified mycobacterial genes are sequence-specifically captured on polymeric beads that are modified with complementary DNA, labeled by SPIONs, and detected by NMR. The platform could detect *M. tuberculosis* and identify drug-resistance strains from sputum samples within 2.5 h. The investigators also developed a similar system that uses rRNA as a target marker for NP labeling.[83] The approach used a universal and specific nucleic acid probes that detects 16S rRNA, which is abundant in and common to many bacterial species. The device was sensitive enough to detect as few as 1–2 E. coli bacteria in 10 ml of blood and accurately estimate bacterial load.

Many groups have also utilized small molecule functionalized NPs to effectively label bacteria. One group developed a magnetic glyco-NP based system that could detect *E. Coli* strains in 5 minutes as well as enabling up to 88% removal from the sample by exploiting

the bacterial interaction with carbohydrates on mammalian cell surfaces (Figure 5).[8485] Another group reported the use of vancomycin-modified SPIONs in a magnetic capture assay for various Gram-positive and Gram-negative bacteria.[86] They also demonstrated that as size and ligand coverage on the NP increases, the time required for efficient labeling to the bacteria with the NPs decreases.

Optical biosensing of bacteria has also been employed using both metallic NPs and QDs. The bio-barcode assay approach[49] provides amplification and the possibility of simultaneously detecting many different targets in one sample. Using this method, *Bacillus subtilis* double-stranded genomic DNA was detected at a 2.5-fM concentration.[87] Similarly, *Salmonella enteritidis* was detected a0.2 fM.[88] QDs have also been used as pathogen sensors. Edgar et al. reported a technique that combined *in vivo* biotinylation of engineered host-specific bacteriophage and attachment of the phage to streptavidin-coated QDs.[89] The method provides specific detection of *E. coli* among several different bacterial strains and can detect as few as 10 bacteria/mL of the experimental samples.

4.1.3 Nanoparticles for cell detection and separation—Efficient isolation of specific cells from complex mixtures is necessary for biological research and for many biological applications. NPs have been explored as sensitive tools for the detection of specific cell types and cells found in low frequency. One application of interest has been the detection and capture of circulating tumor cells (CTCs). CTCs can aid in the understanding of the biology of cancer metastasis and have been described as a strong prognostic biomarker for overall survival in patients with metastatic breast, colorectal, and prostate cancer.[9091]

NP immunomagnetic techniques are some of the more commonly used methods for the identification and capture of CTCs. These techniques involve the use of magnetic NPs to target and isolate CTCs using a ligand-receptor based mechanism. Currently, the CellSearch device, which uses an immunomagnetic technique, is the only FDA-approved test for CTC assessment.[9291] CellSearch is based on a positive epithelial cell adhesion molecule (EpCAM) selection of the CTCs and utilizes iron NPs coated with a polymer layer carrying biotin analogues and conjugated with anti-EpCAM, for the capture of CTCs *in vitro*. This system can also be used for the labeling and identification of leukocytes using an anti-CD45-APC as the NP targeting ligand. Recently, a group also demonstrated that IONPs functionalized with anti-HER2 or anti-HER2/neu could be used to separate 73.6% of HER2/neu over-expressing cancer cells that were spiked in 1 mL of blood.[93] The receptor-ligand interactions resulted in the preferential capture of the cancer cells. In another preclinical study, *in vivo* CTC detection approach was achieved using PEGylated magnetic NPs.[94] Investigators dually targeted CTCs *in vivo* with magnetic NPs conjugated with plasminogen activator (uPA) and folate-targeted nanotubes for subsequent detection with photoacoustic flow cytometry.

The incorporation of polymers, which can enable targeted detection and surface capturing, to other organic and inorganic NP platforms can potentially lead to novel NP sensors for CTC detection.[95] Recently, a group used targeted polymer coated gold NPs and the SERS technique to directly measure CTCs in the presence of white blood cells (Figure 6).[9697] EGF

peptides were conjugated to the polymer coated gold NPs that were encoded with QSY reporters. The NPs successfully identified CTCs in the peripheral blood of 19 patients with squamous cell carcinoma of the head and neck, with a sensitivity range of 1 to 720 CTCs/mL of whole blood.

Other techniques for identification and separation of cells include the use of biomimetic nanotechnology, which takes advantage of naturally occurring processes. The Hong group has investigated biomimetic approaches to develop microfluidic devices for the identification and separation of cells. These devices exploit the natural process of cell rolling, which results from adhesive interactions between selectin molecules expressed on the endothelial venules and glycoprotein receptors on the cancer cells-[98] -Recently, the cell rolling process using E-selectin was applied to CTC detection for enhanced surface sensitivity and specificity.[100] Seventh-generation (G7) poly(amidoamine) (PAMAM) dendrimers were also used to engineer cell capture surfaces for simultaneous binding of multiple ligands to multiple receptors (multivalent binding). The biomimetic combinations of dendrimer-mediated multivalent effect and cell rolling significantly enhanced the surface capture of CTCs.

4.2 Nanoparticles as imaging agents

NPs have been explored as novel labels and contrast agents in molecular imaging. The unique properties of NPs can enable sensitive and specific monitoring of molecular targets as well as of cell responses associated with diseases such as cancer and cardiovascular diseases.[101]

4.2.1 Nanoparticles for targeted imaging—NPs have many promising attributes for targeted imaging. First, NPs can deliver a large number of imaging agents at a time due to their surface area, allowing for improvement in sensitivity.[109] Second, NPs may passively target tissues *in vivo* via the EPR effect or be targeted to accumulate at sites where the molecular target is expressed, increasing the local concentration of contrast agents. The high capacity for NP modification enables their use as amplifiers for *in vivo* imaging. Finally, they can deliver several different types of imaging agents to perform multimodality imaging.

Inorganic NPs such as QDs are among the most promising fluorescent labels for cellular imaging. QDs can emit light of specific wavelengths and also be tuned to emit in the NIR region of the spectrum, in which tissue autofluorescence is reduced and excitation light penetration increased.[110] Monofunctionalized QDs have been used to track individual proteins in cells[111] and for receptors involved in cell movement during development and metastasis.[112] QDs have been used for imaging of specific tumor biomarkers, such as the use of aginine-glycine-aspartic acid (RGD) peptide-conjugated NIR QDS for the targeting of integrin $\alpha_v\beta_3$.[113] Targeted QDs have also been studied for their capability for multiplex imaging, which involves simultaneous imaging of many molecular targets using different QDs that use different emission wavelengths. Recently, QDs have been applied for multiplex molecular imaging of lymph nodes[114], embryonic stem cells[115], as well as tumor cells and vasculature.[116117]

Gold NPs have been studied as a non-invasive modality for *in vivo* cancer imaging using small organic molecules as near-infrared (NIR) SERS reporters. Recently, antibody conjugated gold NPs have been used in conjunction with a sensitive and stable cyanine reporter that developed and screened from a combinatorial library of SERS reporters for the detection of HER2-positive tumors in xenograft models.[118] Other recent *in vivo* imaging efforts have been directed towards the use of several targeted SERS gold NPs for multiplexed imaging in mouse models.[119120]

One of the more well studied NP platforms for molecularly targeted imaging are magnetic NPs. Magnetic NP imaging systems have shown potential for real-time visualization of biological events, such as cell migration/trafficking,[121122123]enzyme activities[124], and other biological interactions at the molecular and cellular level.[25] Magnetic NPs have also shown promising use as contrast agents in magnetic resonance imaging (MRI), a biomedical technique based on nuclear magnetic resonance of various interacting nuclei. SPIONs, formed from iron oxide crystals coated with dextran or carboxydextran, is a widely used MRI contrast agent for cancer imaging.[20] When injected into patients, SPIONs have been shown to remain the tumors 24 hours after injection compared with 1 hour for gadolinium-based MR agents.[125] This difference is due to easier uptake of the NP by tumor tumors and lower diffusivity of the NP out of the tumor.

Many studies have investigated the use of SPIONs for the targeted detection of tumors and their metastases,[126127128129] and apoptotic tumor tissues.[24] SPIONs have also been targeted to cancer cells without using targeting ligands. In one study, it was demonstrated that a recombinant human heavy-chain ferritin protein shell containing IONPs targeted tumor cells overexpressing transferrin receptor 1.[130] The iron oxide core also catalyzed the oxidation of peroxidase substrates in the presence of hydrogen peroxide to produce a color reaction that is used to visualize tumor tissues. In another study, investigators assembled SPIONs and targeting peptides on a modified viral scaffold to increase the number of SPIONs reaching tumor cells.[129] Glutamic acid residues were introduced into the protein coat of M13, a virus that infects bacteria. The negative charged residues promoted the electrostatic assembly of NPs along the M13 coat's filamentous structure. The viral coat was also rendered to display a peptide that targets SPARC glycoprotein, which is overexpressed in various cancers. Compared to traditional approaches where NPs are directly functionalized with targeting ligands, this approach may amplify the contrast when performing MR imaging.

Novel NPs with advanced magnetic properties have also been pursued for visualizing biological events. One such class is metal-doped ferrite NPs with a composition of MFe_2O_4, where M is +2 cation of Mn, Fe, Co or Ni, to tune specific magnetic properties.[127] $MnFe_2O_4$ NPs were found to be non-toxic *in vitro* and possess the highest magnetic susceptibility, suggesting that they may make better MRI probes. When these NPs were conjugated with antibodies, they showed enhanced MRI sensitivity for the detection of cancer markers. Other NP platforms have been combined with SPIONS including dendrimers (magnetodendrimers)[123] and liposomes (magnetoliposomes)[131]. These SPIONs have been used for applications such as monitoring the migration of cells or visualizing bone marrow *in vivo*.[132]

4.3 Nanoparticles as Delivery Vehicles

4.3.1 Delivering siRNA for biological studies—RNA interference (RNAi) is an important biological tool for use in cell culture and in living organisms. It is traditionally used to study gene functions because it allows targeted degradation of mRNA after the introduction of sequence-specific double stranded RNAs into cells. However, effective siRNA delivery can require overcoming many biological obstacles: 1) difficulty entering the cell because of high molecular weight and negative charges, 2) degradation by nucleases within the cell, 3) targeting to the appropriate cell compartment and 4) rapid clearance and instability *in vivo*.[133134135] Therefore, to realize the potential of RNAi, efficient and biocompatible delivery systems are needed.

NPs offer a potential solution to the challenges in siRNA delivery. Cationic lipid or polymer NPs have been used to transport anionic nucleic acids into cells due to their ability to form a condensed complex with nucleic acids.[136] This stabilizes and protects them from enzymatic degradation. Cationic materials can also help NPs escape sequestration in endosomes/lysosomes. Groups such as the nitrogens in the cationic polymer polyethyleneimine (PEI), which become protonated in the pH environment of the endosome/lysosomes, can facilitate endosomal escape by increasing Cl^- influx in response to protonation at acidic pH. The result is an increase in osmotic pressure and swelling, which leads to organelle burst and delivery of the siRNA NPs. This phenomenon is referred to as the “proton sponge effect”. However, a recent study showed that siRNA delivery in a cationic lipid NP system was substantially reduced as approximately 70% of the internalized siRNA were recycled to the extracellular media due to the exiting of the lipid NPs from late endosomes/lysosomes.[137] Thus, siRNA delivery might be improved by designing novel NP vehicles that can escape the recycling pathways. Active targeting methods of non-endocytic uptakes of NP delivery of nucleic acids have been also been explored using fusogenic peptides and cell penetrating peptides.[138] To better understand siRNA NPs interactions with biological systems, siRNA probes have been used to study intracellular trafficking[139] as well as assembly and disassembly of siRNA NPs.[140]

Recently, NPs have been used to deliver siRNA to silence genes in immune cells since these cells can have pivotal roles in homeostasis and disease.[141] In one study, NPs were used to selectively silence Cyclin D1 (CyD1), a cell cycle–regulatory molecule, in leukocytes *in vivo* to determine the exact roles of the molecule in gut inflammation.[141] NPs were loaded with CyD1 siRNA and functionalized with antibodies to β_7 integrin. The study revealed that these targeted NPs silenced CyD1 in leukocytes and reversed experimentally induced colitis in mice by suppressing leukocyte proliferation and T helper cell 1 cytokine expression. Another recent report also described the use of lipid NPs for the *in vivo* delivery of siRNA to silence disease genes in immune cells.[142] The study demonstrated siRNA-mediated silencing in myeloid cell types of nonhuman primates and established the feasibility of targeting multiple gene targets in rodent myeloid cells. The therapeutic potential was validated using siRNA targeting tumor necrosis factor-α (TNFα). Another study used NPs to explore the immunological mechanisms triggering nonalcoholic steatohepatitis (NASH).[142] The investigators found that TNFα produced by Kupffer cells can trigger the development of NASH through the enhanced production of chemokines IP-10 and MCP-1.

Moreover, TNFα silencing in myeloid cells reduced the chemokine production and prevented the development of NASH, suggesting potential of TNFα as a novel therapeutic target in NASH.

NPs have also been used as a vehicle to deliver siRNA in plant cells to study cellular pathways at the single cell level.[143] One group used amine-conjugated polymeric NPs as vehicles to siRNAs targeting specific genes in the cellulose biosynthesis pathway. They found that *NtCesA-1*, a factor involved in cell wall synthesis in whole plants, also plays an essential role in cell wall regeneration in isolated protoplasts.

4.3.2 Delivering hydrophobic compounds without solvent or excipients—Many biologically active compounds are hydrophobic molecules and are poorly soluble in water. Utilizing such compounds in biological research can be challenging because of their poor solubility in aqueous environments. Current approaches involving using a solvent such as dimethyl sulfoxide (DMSO) or an excipient such as cremophor. However, not all compounds can be solubilized using these solvents and the solvents themselves frequently incur toxicities from living organisms that can complicate the biological experiment. For example, wortmannin, a PI3 kinase inhibitor and a commonly utilized reagent in biology research, requires DMSO for *in vitro* applications. DMSO is known to have its own effects on cells and is also not suitable for *in vivo* applications. One of the strategies to overcome the challenge of delivering these hydrophobic agents is to utilize NPs. Many NPs, especially polymeric NPs, have hydrophobic cores and are well suited for the delivery of hydrophobic agents. The use of NP delivery vehicles not only overcomes the solubility of the active agent, but also protects the agent from the environment until the agent is released from NP. Our own group has demonstrated the proof-of-principle of this approach by developing a NP formulation of wortmannin.[144] We demonstrated that NP wortmannin increased its solubility and improved its stability. We were also able to show that NP wortmannin has the same signaling effects on cells as wortmannin. Moreover, NP wortmannin functioned as an effective and potent therapeutic agent *in vivo* in a mouse model of cancer.

4.3.3 Delivering agents to subcellular organelles—One area of active investigation is delivering various agents to specific organelles. In particular, subcellular availability and accessibility of a target is important in effective delivery of therapeutic and imaging agents.[145] Delivering agents to subcellular organelles can also potentially illuminate certain molecular processes that are still unknown in organelles.

The ability to easily modify and functionalize NPs has resulted in recent interest in using these vehicles to deliver agents to subcellular organelles. Targeted NPs can bind to targets localized on the cell surface and enter the cell through endocytosis. However, if the target is located intracellularly, NPs and their cargo may not be able to reach the target of interest due to intracellular sequestration of the NP or due to the lack of subcellular targeting capabilities. In particular, NPs carrying oligonucleotides need to escape the endosome and then be targeted to be effective. Tools for effective subcellular targeting are emerging for targeted delivery to the nucleus,[146] cytosol,[147] mitochondria,[148] endosomes,[149] and lysosomes.[150] In general, two approaches are being investigated for the design of NPs for subcellular targeting: 1) Passive targeting of NPs to a particular organelle by varying NP

characteristics such as size, shape, and composition.[151] 2) Active targeting of NPs to the organelle of interest by functionalizing NP surfaces with targeting ligands directed towards the organelle. These approaches have been applied with varying levels of success.

Hurdles to successful sub-cellular targeting include biological barriers specific to the target organelle. For example, NPs targeted to the nucleus must enter the cell membrane, escape endosomal/lysosomal pathways, possess a way to interact with the nuclear pore complex, and be small enough to cross the nuclear membrane.[146] Targeting ligands such as the nuclear localization signal (NLS) has been used to enhance nuclear delivery by active transport mechanism.[152] In one study, the localization of NLS conjugated gold NPs at the nucleus of a cancer cell damaged the DNA.[152] On the contrary, in another study, NLS conjugated gold NPs were not able to target the nucleus of cells from outside the plasma membrane because they were unable to enter the cells or were trapped in the endosomes. Instead, NPs conjugated with both NLS and receptor-mediated endocytosis (RME) peptides reached the nucleus.[153]

For NPs targeted to the mitochondria, biological barriers include intracellular transport to the mitochondria and outer and inner mitochondrial membranes and toxicity.[154] Thus far, most studies have primarily developed metal oxide or liposomal NPs for delivery to the mitochondria. Delivery to the mitochondria has also been based on electrostatic interactions between the NP and the organelle. The mitochondria has a negative membrane potential compared to other cellular membranes, which can lead to the accumulation of liphophilic cations.[155] This concept was utilized in the fabrication of stearyl triphenyl phosphonium (STPP) targeted liposomes with anti-cancer agents ceramide[156] and scarleol[157] to target the mitochondria. STPP was chosen since it exhibits both cationic and lipophilic properties. Another group used a polymeric NP system to deliver mitochondria-acting therapeutics to their destination.[158] The NPs were synthesized with a lipophilic triphenylphosphonium (TPP) cation, which is known to cross into the mitochondrial matrix space. Through *in vitro* screening of a library of charge and size varied NPs, the group identified an optimized targeted NP that improved the efficacy and decreased the toxicity for cancer Alzheimer's disease, and obesity compared with non-targeted NPs or the small molecule therapeutics.[158]

4.4 Effects of biology on NPs

Understanding NP interactions with biological systems is necessary in developing effective NPs as sensing, imaging and drug delivery agents. Thus, studies have increasingly focused on the biological effects of NP properties. Properties of NPs such as size, shape, functional groups, surface charge, and composition are all factors that have been shown to affect NP interactions with biological systems *in vitro* and *in vivo*.[102] However, less work has been done to study the effects of biology on NPs. It is known that NPs are rapidly cleared by the cells of the reticuloendothelial system (RES)/mononuclear phagocyte system (MPS) in the body.[103] To extend the circulation time of NPs in vivo, uncharged hydrophilic polymers such as PEG are often conjugated to the surface of particles for "stealthing". Yet, antibodies against PEG can develop after the first administration of PEGylated particles. Understanding the biomolecular interactions of nanoparticles and the MPS is critical for the development of alternative methods for extending NP circulation times in vivo.

Recently, it has been shown that the uptake of NPs by cells *in vitro* can be influenced by cell cycle phase.[104] The study found that NPs internalized by cells are not exported from cells, but are split between daughter cells when the cell divides. The results may have implications on clearance or accumulation of NPs *in vivo*. Another recent study demonstrated that global immune status, such as the balance of Th1-Th2 cytokines and M1-M2 macrophages, can also affect the NP clearance process.[105] The investigators showed that mouse strains that are prone to Th1 immune responses cleared NPs at a slower rate than Th2-prone mice. Macrophages isolated from the Th1 strains also took up fewer particles *in vitro* than macrophages from Th2 strains. Results were confirmed in human monocyte–derived macrophages, suggesting that global immune regulation may affect NP clearance in humans.

Due to their small sizes and the EPR effect, NPs are often passively or actively targeted to cancer tumors.[106] Yet the clearance and distribution of NPs can also greatly depend on the tumor vasculature. Jain and colleagues have suggested that leaky and poorly organized blood vessels of cancerous tumors can result in an increase in the interstitial fluid pressure inside tumors, reducing blood supply to them and thus impairing delivery of agents to the tumors.[107] They showed that repairing the abnormal vessels in mammary tumors, by blocking vascular endothelial growth factor receptor-2, improves the delivery of smaller NPs (12 nm), while hindering the delivery of larger (125 nm). The role of other proteins such as TGF-β in normalizing tumor vasculature for NP delivery in tumors has also been explored.[108] TGF-β blockade was found to significantly decrease tumor growth and metastasis in mice. It also increased the recruitment and incorporation of perivascular cells into tumor blood vessels, increased the fraction of perfused vessels, and decreased the collagen I content of the tumor interstitial matrix. The investigators found that as a result of the vessel and interstitial matrix normalization, TGF-β blockade improved the intratumoral uptake of NP therapeutics, leading to better control of tumor growth.

4.5 Nanoparticles to study biological processes

The unique properties of NPs have enabled their use as promising tools to study biological processes. Many innovative techniques using NPs are being developed to activate cell signaling pathways, to induce protein production, and to improve upon current techniques used in molecular and cellular biology research. NPs such as QDs have been extensively studied for many biological applications that use fluorescence. Some of its uses include immunostaining of fixed cells and tissues, membrane proteins and cytoskeleton filaments.[31, 159] Recently, QDs have also been used to visualize the molecular dynamics of individual molecules in live cells. One group visualized EGF-bound receptor movements by tagging a small fraction of individual EGFR molecules with a conjugate of one CdSe QD linked to one anti-EGFR antibody Fab fragment (antiEGFR–Fab).[160] The individual antiEGFR–Fab-QD-bound EGFRs (EGFR–Fab-QD) were then visualized by total internal reflection fluorescence microscopy.

NP platforms can also enable the local perturbation of protein activities in cells at a subcellular scale. In particular, magnetic NPs can be coated with a biocompatible surface layer that can be functionalized with ligands that target specific cell-surface receptors, which then can be activated remotely by applied magnetic fields. In a recent study, investigators

used this approach to study how NP mediated activation of specific signaling pathways can lead to changes in cellular responses.[161] The magnetic NPs are attached with active signaling proteins, and can be displaced by magnetic forces into different locations of the cell. Once these protein-conjugated NPs are inserted into the cells, they bind partner proteins to their surfaces and locally stimulate signal transduction pathways. This strategy was applied to members of the Rho-GTPases, a set of molecular switches known to regulate cell morphology. NP mediated Rac1 signal was found to induce actin polymerization in protrusive areas of cells while no NP induced actin polymerization was observed in the other areas of the cell, suggesting that Rac1 associates with another partner to polymerize actin in the regions of high membrane activity. The investigators demonstrated that the NP-mediated activation of signaling pathways could also lead to a local alteration of cellular morphology and remodeling of the actin cytoskeleton. Thus, the strategies used in this study could be used to enhance understanding of how other biomolecules are spatially modulated and integrated at the cellular level.

The use of magnetic NPs for controlling cell signaling pathways was demonstrated in another study by Cheon and colleagues.[162] The group showed that functionalized magnetic NPs could turn on apoptosis cell signaling when a magnetic field is applied. The magnetic switch developed consisted of zinc-doped IONPs conjugated with a targeting antibody for death receptor 4 (DR4) of DLD-1 colon cancer cells. When a magnetic field is applied to aggregate DR4-conjugated IONPs bound to cell surface receptors, an apoptosis signaling pathway is promoted. This magnetic control of apoptosis was demonstrated *in vivo* using zebrafish as a model organism. IONPs can also be used to activate cells and remotely regulate protein production.[163164] Stanley and colleagues used anti-His conjugated IONPs to bind to a modified epitope tagged TRPV1 channel. The temperature sensitive TRPV1 channel is activated with local heating of bound anti-His IONPs, resulting in activation of a Ca^{2+}-sensitive promoter that stimulates the synthesis and release of bioengineered insulin. This result along with lowered blood glucose was confirmed *in vivo* with mice expressing the bioengineered insulin gene. The investigators further showed that cells can be engineered to synthesize genetically encoded ferritin NPs and inducibly release insulin. The use of IONPs in noninvasive techniques for cell manipulation potentially provides a useful tool for basic biology research.

Other inorganic NP platforms have also shown potential as tools to regulate cellular activities with spatial and temporal control. Recently, infrared-absorbing gold NPs were used as optical switches of gene interference and were remotely controlled using light.[165] The technique involved functionalizing the NPs with double-stranded oligonucleotides and at specific times and intracellular locations, NIR illumination was used to photothermally heats the gold NPs, causing the double-stranded oligonucleotides to denature at their melting temperature and the antisense oligonucleotides to be released from the carriers.

On a macroscopic scale, the spatial organization of cells in cell culture can be important in basic biology research. Since cellular activities in conventional 2D cell culture differ from those found *in vivo*, many efforts have focused on developing 3D cell culture for a more physiologically relevant microenvironment. Recently, Pasqualini and colleagues reported an innovative approach to 3D tissue culture based on the magnetic levitation of cells with a

hydrogel consisting of gold NPs, IONPs, and M13-derived phage particles that display integrin-targeting ligands.[163] By spatially controlling the magnetic field, the authors were able to control the geometry of the cell mass and produce concentrated clustering of different cell types in co-culture. They also found that magnetically levitated human glioblastoma cells showed similar protein expression profiles to those observed in human tumor xenografts. The results indicate that magnetic levitation of cells may be a useful method for recapitulating *in vivo* protein expression without the use of a specific medium, engineered scaffolds or matrices.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Nanotechnology and NPs have a wide range of applications in biology. As mentioned above, nanotechnology enable the creation of devices on the same scale as individual cells and biomolecules, creating an unique approach to imaging, sensing, drug delivery and characterizing basic biological processes. Since the interest in biological application for NPs is very recent, there is reason for high optimism for more innovative and exciting applications of NPs in biology.

While a significant number of genes and proteins integral to biological processes have been identified, the manner in which many of these biological units and processes assemble and integrate still remains unknown. Biological studies that employ NP techniques can provide novel insights into cell functions and molecular processes involving complex signaling pathways. NPs can also be used to help understand how these processes can be constructively modified and how various components of a cell work together to accomplish a task. For instance, biological processes such as apoptosis, cell division, and stem cell fate are modulated by mechanical cues and changes in their physical environment. In understanding molecular mechanisms of mechanotransduction, NP analytical devices can be used to measure and manipulate interactions of biological molecules and cells to determine physical relationships between cellular components and understand how cells respond to mechanical forces and chemical cues.

NP monitoring and detection techniques can potentially aid in understanding the basis of biochemical pathways involved in disease and injury. NP sensors can enable the identification of biological molecules not addressable by current assays. Targeted NP systems can bind or react to the presence of these molecules with high affinity and selectivity, amplifying their signal for detection and molecular imaging. Furthermore, NPs can help overcome limitations associated with conventional delivery methods used in biological research such as insolubility and instability of hydrophobic compounds under aqueous conditions and nonspecific targeting. With significant progress in studying biological systems at the nanoscale and an integration of proteomics and genomics with nanotechnology, a deeper understanding of cell function and processes can be obtained that can in turn help in the design of novel nanoscale tools that may bring molecular systems back to normal operations after their function has been perturbed by disease. It is also important to note that as nanotechnology methods matures, there is less technical barrier for interested biologists to acquire NP techniques/platforms and adopt them into their own research. Currently, there are various commerical NPs available for use as contrast agents in

imaging modalities such fluorescence imaging and magnetic resonance imaging and for detection of low concentrations of analytes. However, as mentioned previously, NP properties such as size, shape, functional groups, surface charge, and composition are all factors that may affect NP interactions with biological systems *in vitro* and *in vivo*. Thus, careful evaluation of effects such as toxicity induced by different types of NP formulations in biological systems are important in employing NPs for biological applications. The full utilization of NP technology in biological research will require chemists and nanotechnologists to standardize and simplify NP fabrication techniques. It will also involve nanotechnology researchers actively engaging and collaborating with biologists to identify new areas of application and synergy.

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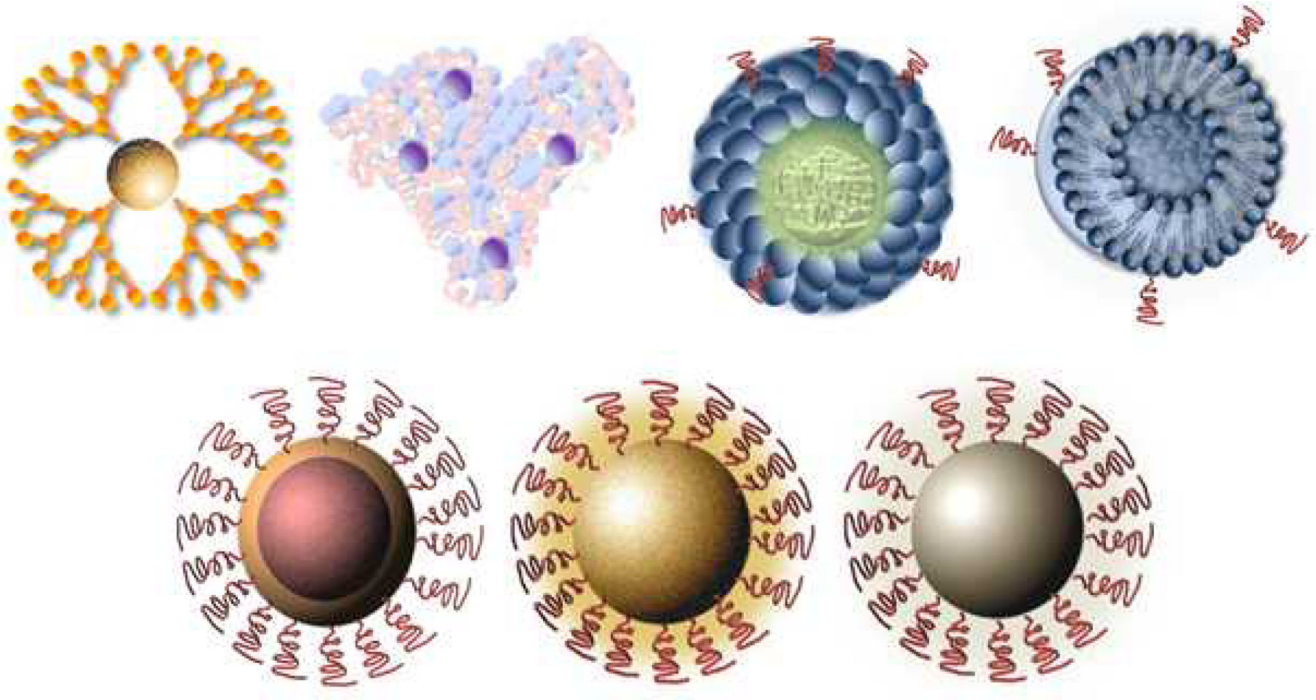

Fig. 1.
Schematic representation of different types of nanoparticles.

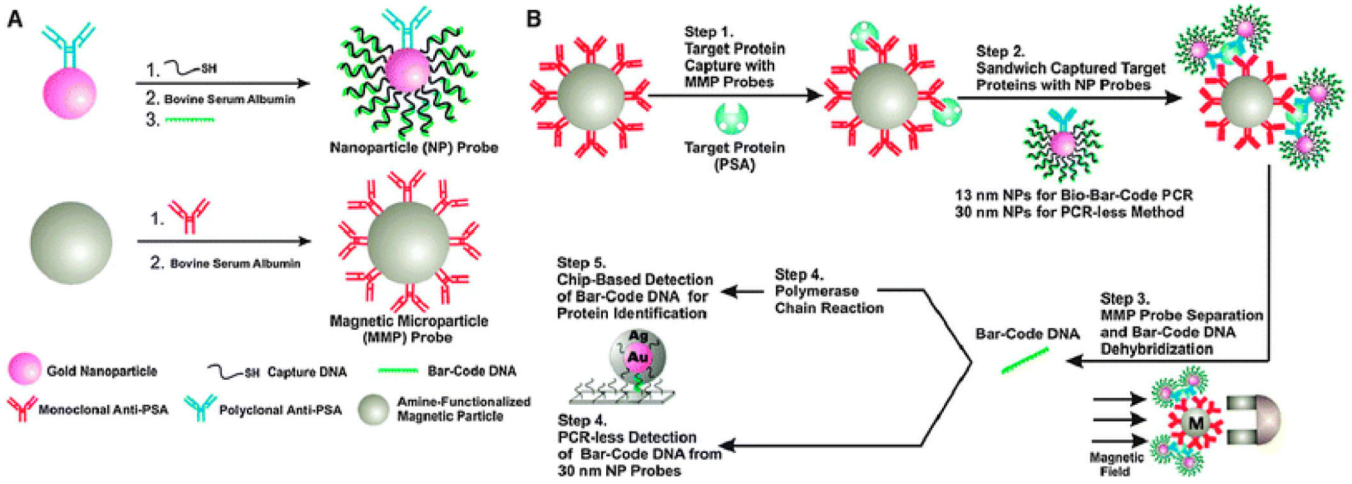

Fig. 2.

The bio-barcode method. A. Development of the NP probe. B. Method of detection using prostate specific antigen-conjugated gold NP probes. Reproduced from Ref. 55.

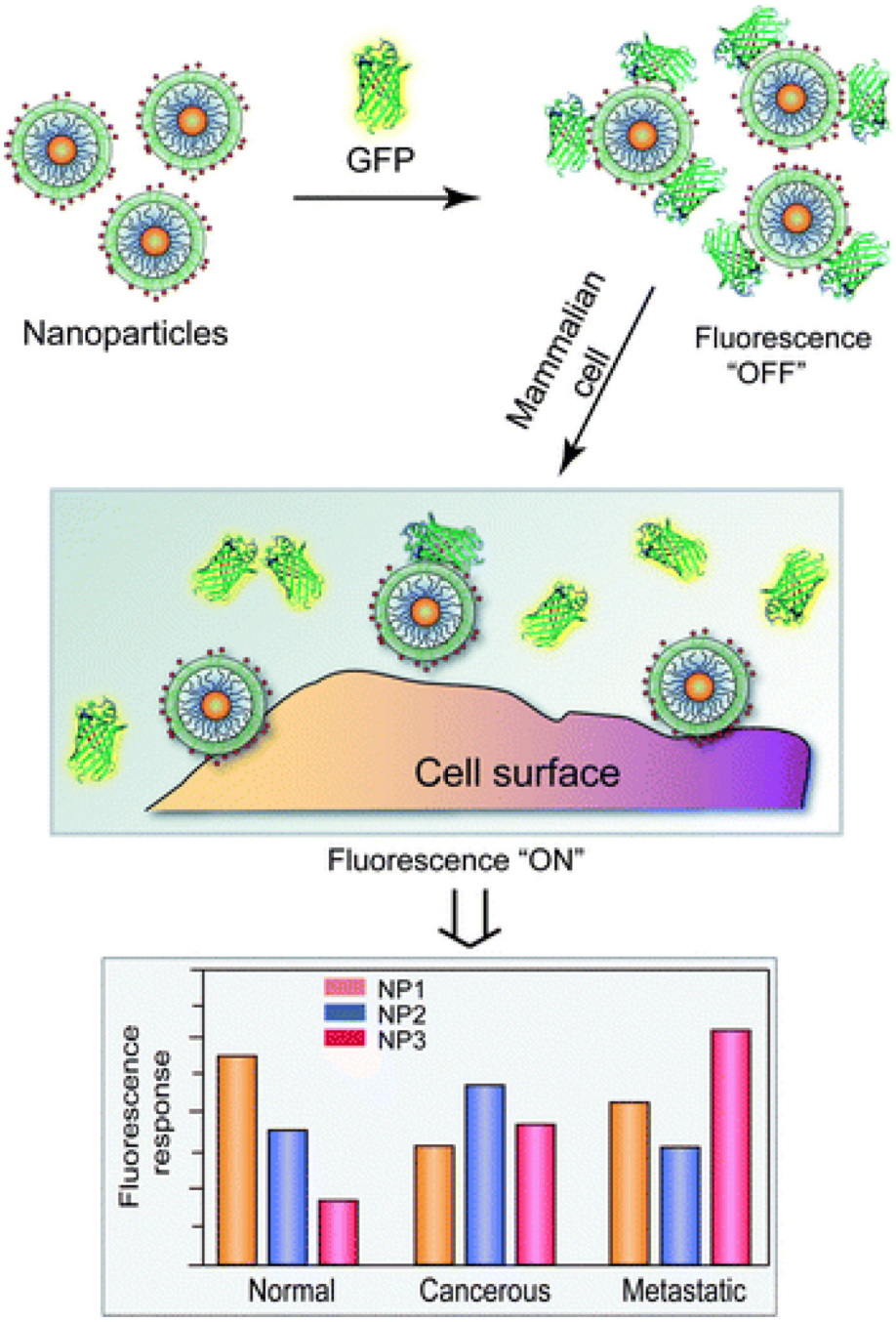

Fig. 3.

Interactions between NP-GFP complexes and cell surfaces generates different fluorescence patterns. Reproduced from Ref. 60.

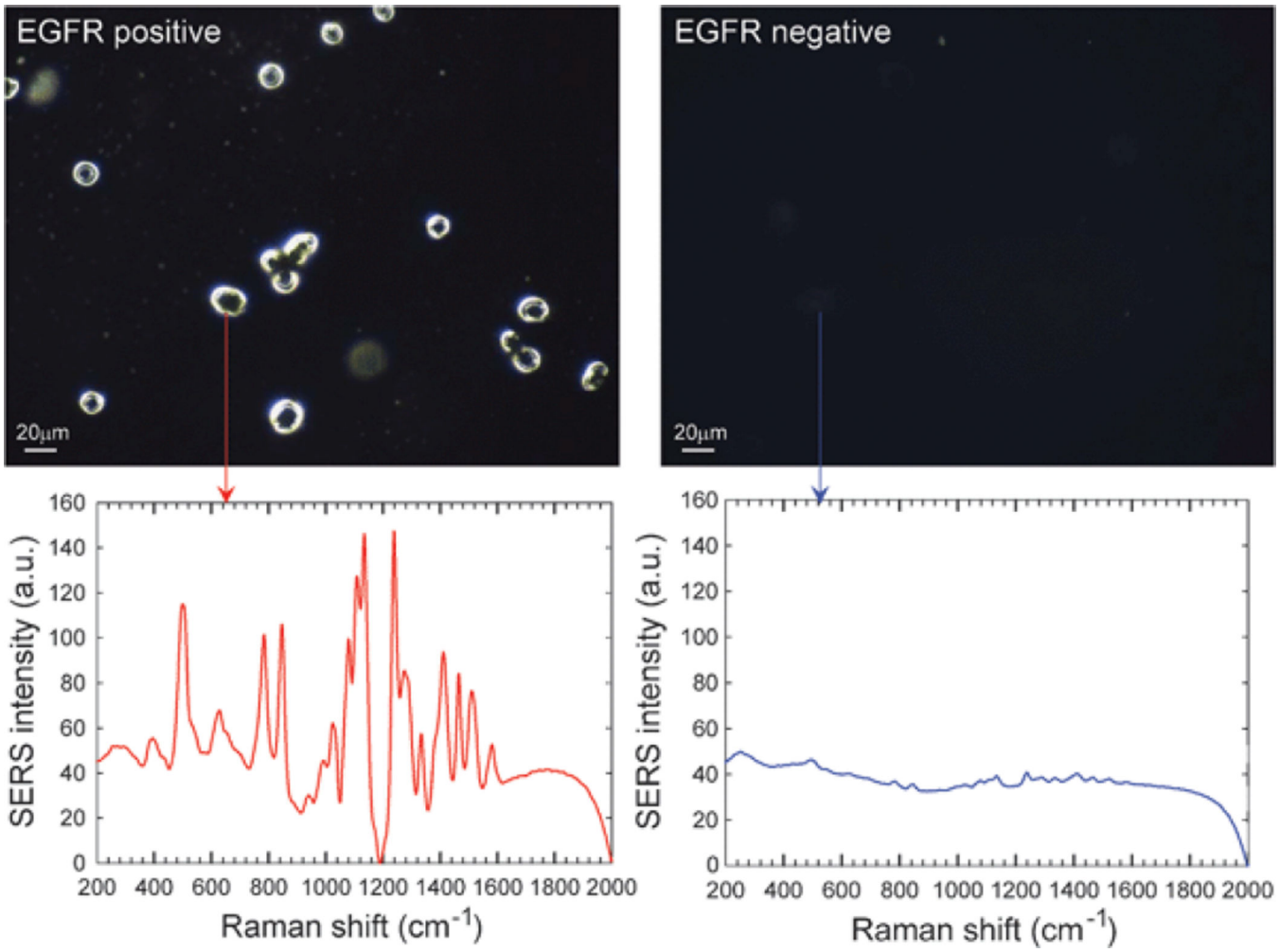

Fig. 4.

Surface-enhanced Raman scattering (SERS) spectra and the correlated surface plasmon imaging of single cancer cells tagged with ScFv-conjugated gold NPs. Reproduced from Ref. 66.

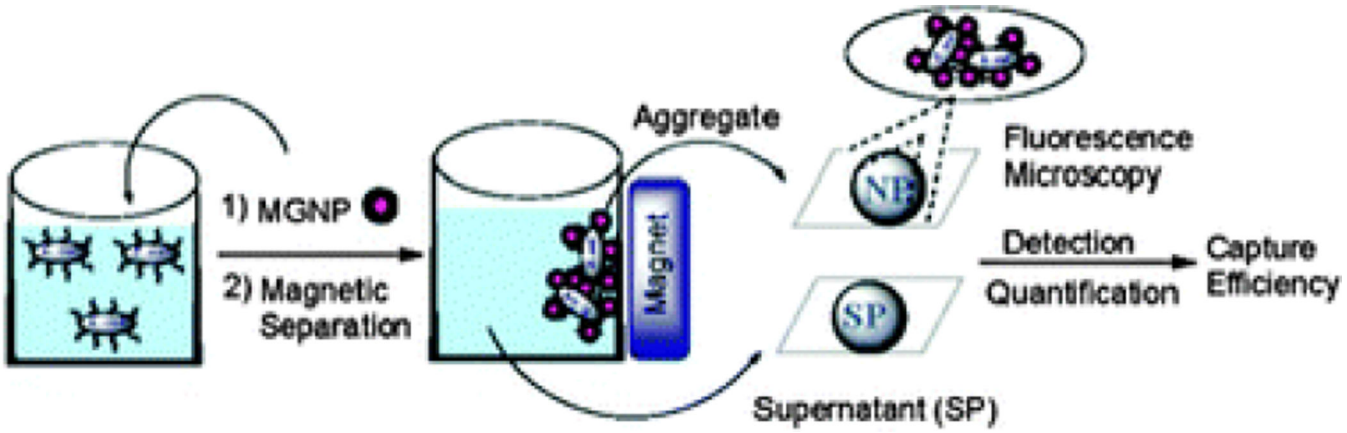

Fig. 5.
Depiction of bacteria detection by magnetic glyco-NPs (MGNPs). Reproduced from Ref. 85.

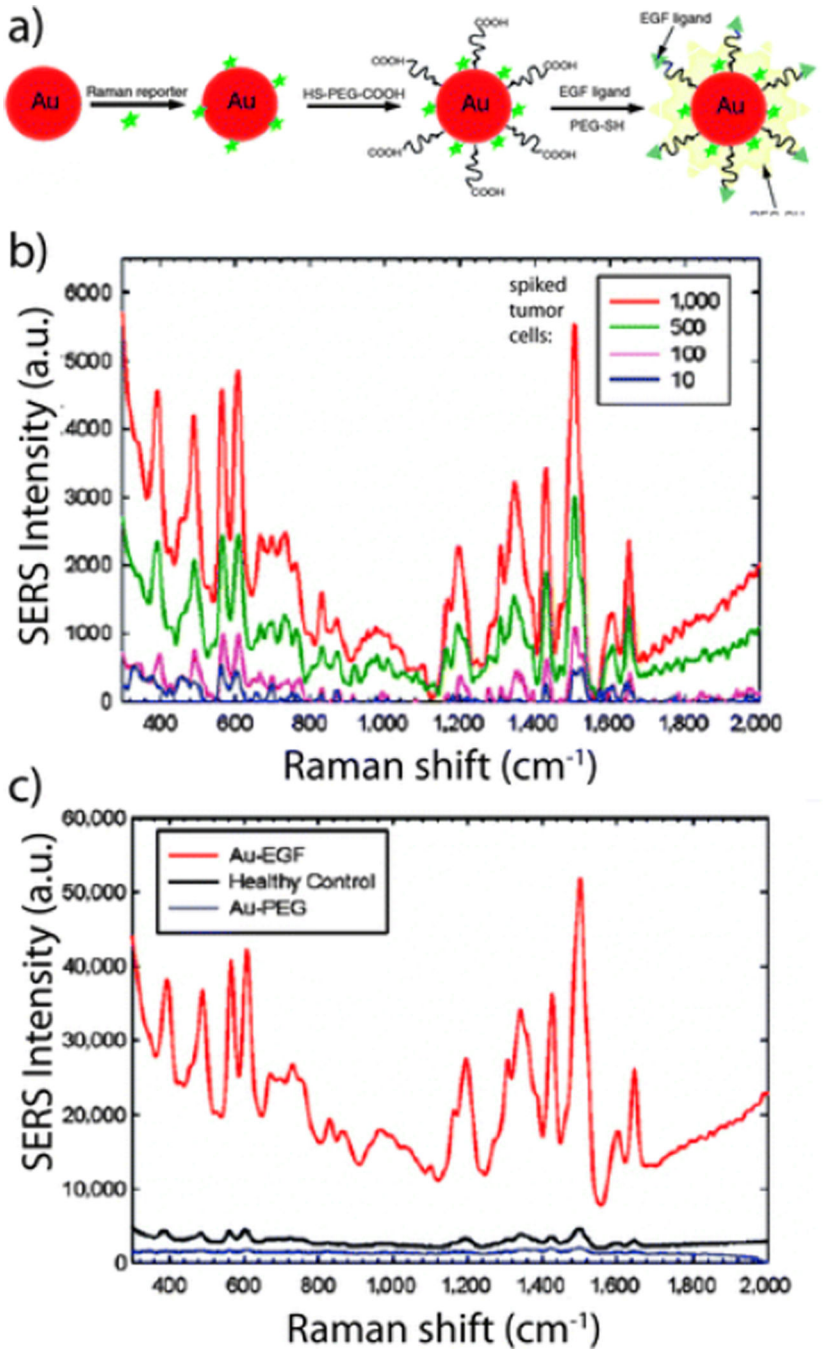

Fig. 6.

Enhance surface-enhanced Raman scattering (SERS) detection of circulating tumor cells. A) Schematic of EGF peptide-conjugated NPs. B) SERS spectra of different numbers of cancer cells spiked into white blood cell sample. C) SERS spectra of a blood sample incubated with targeted and non-targeted NPs. Reproduced from Ref. 97.