

Dye-Doped Fluorescent Nanoparticles in Molecular Imaging: A Review of Recent Advances and Future Opportunities

YADOLLAHPOUR ALI*, REZAEI ZOHRE, JALILIFAR MOSTAFA and RASHIDI SAMANEH

Department of Medical Physics, School of Medicine,
Ahvaz Jundishapur University of Medical Sciences, Golestan Blvd., Ahvaz, Iran.

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ABSTRACT

Molecular imaging (MI) is an in vivo assessment of characterization and quantitatively measurement of biological processes at the molecular level. Determination of pathologies of malfunctioned tissues without invasive biopsies or surgical procedures, early detection, monitoring of treatment process and visualization of cell trafficking are advantages of this approach. One example of basic requirement of MI is high affinity molecular probe that acts as the source of image contrast. Recent advances in nanotechnology have developed the use of nanoparticles as MI probe. Optical molecular imaging is one of the main categories of molecular imaging with great potentials for in vivo cell trafficking. Fluorescent nanoparticles are a major group of nanoparticles in optical molecular imaging. Dye-doped, quantum dots and upconversion particles are three classes of fluorescent nanoparticles. This paper reviews the basic principles of molecular imaging based on nanoparticles focusing on the optical molecular imaging. The characteristics of dye-doped nanoparticles, their as well as of that are reviewed in this paper.

Key words: Molecular imaging, fluorescent nanoparticles, dye doped nanoparticles, quantum dots, upconversion particles.

INTRODUCTION

Throughout the history of medicine, two main concepts of disease managements have been dominant: Ontological and ontological concept. The ontological concept defines a disease as an entity that is independent, self-sufficient, which develops a regular course with a natural history of its own. The physiological concept views disease as a deviation from normal physiology or biochemistry[1]. Claude Bernard hypothesized that the same biological processes that make life possible are also involved in disease. In other words, the laws of disease are the same as the laws of life. All the organs and tissues of the body perform functions that help maintain homeostasis[2]. According to the cell theory proposed by Theodor Schwann, all living organisms are consisted of discrete cells [3-5]. In 1858, Rudolph Virchow correlated disease with cellular abnormalities[6]. Although most diseases are

triggered by a cell injury [7], diagnosis and treatment often do not start until the onset of the symptoms of a disease, which may be a considerable time after the beginning of biochemical changes on the cellular level. Generally, by the time the diagnosis is made, the disease will have progressed to a stage where therapeutic efforts are more difficult and costly, and the chances for cure are smaller [8, 9].

Watson and Crick's model of DNA in 1958 initiated a research era where biological and physical scientists would strive to identify the genetic code and its regulated expression that is the basis of development and maintenance of phenotypic function of all cells of an organism[10]. Indeed advances in sequencing the human genome and the genomes of many other organisms provided an enormous amount of DNA sequence data and genomic information that transformed the way we can study living organisms[11]. As a result, physical,

biological and medical sciences are working together to identify fundamental errors of disease and develop molecular corrections for them. The name given to this broad field of endeavor is "Molecular Medicine"[12].

Molecular imaging is an *in vivo* characterization and quantitatively measurement of biological processes at the cellular and molecular level [13, 14]. In this field, instead of diagnosing and classifying disease by symptoms or systemic changes, tests are being developed for the characteristic biological and biochemical markers and processes which occur in various types of disease[15]. The main advantage of an *in vivo* molecular imaging is its ability to determine pathologies of diseased tissues without invasive biopsies or surgical procedures[16]. Tumors may be spatially and temporally heterogeneous in terms of gene expression[17], metabolism[18], hypoxia[19], angiogenesis[20], cell proliferation[21], apoptosis[22, 23], and other phenotypic features, but this technique can help investigators to better understand these features[24]. These features are important to tumor detection, characterization, staging, prognosis assessment, treatment planning, and early treatment monitoring, as well as for monitoring of cell trafficking and new drug development[25, 26]. Molecular imaging for performance, have two basic requirements include: (i) high affinity molecular probes with the ability to overcome biologic delivery barriers and (ii) a sensitive, fast, high spatial and temporal resolution imaging modality to detection this probe[27]. Molecular imaging probes that provide imaging signal are referred by many different names such as molecular beacons, reporter probe, tracers, smart probe, activatable probe, contrast agent, and nanoparticles[28]. The rapid growth of nanotechnology and nanoscience could greatly expand the clinical opportunities for molecular imaging[29]. The basic rationale is that nanometer-sized particles have functional and structural properties that are not available from either discrete molecules or bulk materials[30, 31]. When conjugated with biomolecular affinity ligands, such as antibodies, peptides or small molecules, these nanoparticles can be used to target malignant tumors with high specificity[32]. Structurally, nanoparticles also have large surface areas for the attachment of multiple diagnostic and

therapeutic agents. Recent advances have led to the development of biodegradable nanostructures for drug delivery[33]. Imaging modality is one of the most important requirements of a molecular imaging technique [34]. Different modalities can be used in molecular imaging including single photon emission computed tomography (SPECT), positron emission tomography (PET), magnetic resonance imaging (MRI), ultrasound (US) and optical imaging. Sensitivity, spatial resolution, temporal resolution, depth of signal penetration and cost of these modalities are different[35]. Between all of them, optical imaging is the safest method. Possibility of real-time imaging, fast data acquisition (minutes), relatively high spatial resolution and low cost are the most important advantages of this technique[36, 37]. Optical molecular imaging is an imaging discipline that measures light released from either endogenous sources or exogenously administered agents that, encoded within its signal, bears information about biological processes on a microscopic scale[38]. For improved signal-to-background ratio (SBR) and targeting of specific biological activity, this imaging technique relies on the excitation and detection of fluorescence from an exogenous contrast agent [11]. Exogenous optical agents can be categorized into three classes: (i) organic dyes (ii) quantum dots and upconversion nanoparticles[39]. This review is divided into three main sections. In the each section, we review the synthesis techniques, properties and molecular applications of one category of optical nanoparticles.

History of nanotechnology and emerging of fluorescent nanoparticles

The first use of nanotechnology was in a speech by Richard Feynman in 1959, entitled "there's plenty of Room at the Bottom". Feynman suggested a means to develop the ability to manipulate atoms and molecules directly, by developing a set of one-tenth-scale machine tools. These small tools would be used to develop and operate a next generation of one-hundredth-scale machine tools, and so forth. As the sizes get smaller, it would be necessary to redesign some tools because the relative strength of various forces would change. Gravity would become less important, surface tension and van der Waals attraction would become more important[40]. The term "nanotechnology" was first defined by Tokyo Science University, Norio Taniguchi in a 1974 paper as follows:

“Nanotechnology” mainly consists of the processing of, separation, consolidation, and deformation of materials by one atom or one molecule[41]. the first controlled release polymer system of macromolecules was described in 1976[42]. In the 1980s the idea of nanotechnology as deterministic, rather than stochastic, handling of individual atoms and molecules was conceptually explored in depth by Drexler. His vision of nanotechnology is often called molecular nanotechnology (MNT)[43]. Nanotechnology and nanoscience got a boost in the early 1980s with two major developments: the birth of cluster science and the invention of the scanning tunneling microscope (STM). This development led to the discovery of fullerenes in 1985[44]. In the early 1990s Huffman and Kraetschmer, of the University of Arizona, discovered how to synthesize and purify large quantities of fullerenes[45]. Today, we know there are two different types of fluorescent imaging contrast that can be used in optical molecular imaging: endogenous and exogenous agent. Endogenous agents to produce the optical signal, work by enzyme-mediated process in cells and tissues. Two important examples for endogenous probes include: fluorescent proteins[46] and luciferin/luciferase systems[47]. The most important limitations of fluorescent proteins are their need to genetical modification of targeted cells and low emission wavelength (510 nm). Furthermore, luciferin/luciferase systems suffer from inhomogeneous scattering and limited light penetration[48]. The exogenous contrasts which are inserted into the biological system can be classified into three different types: (1) dye doped, (2) quantum dots (QDs) and (3) upconversion particles.

Dye-doped Nanoparticles

Conventional organic dyes are one of the most important optical agents. Fluorescein iso thiocyanate (FITC), carboxy fluorescein diacetatesuccinidyl ester (CFSE) and IRG-023 Cy5 are some examples of the organic dyes. These materials have several drawbacks, such as: chemical instability, rapid photobleaching, sensitivity to pH and biodegradation in biological environment. Therefore we cannot use them for long term cell tracking. In addition, dyes are excited by UV-Visible light that has low penetration depth, and low signal to noise ratio due to autofluorescence. Because of these drawbacks, Organic dyes are not suitable for

simultaneous multicolour imaging[49]. Although, the problem of autofluorescence can be resolved by using NIR emitting dyes such as cyanine particles, these particles have low quantum yield and poor photostability[50]. Therefore, conventional dyes are not an ideal candidate for optical imaging. To overcome the limitations of conventional dyes, Santra *et al* have developed a dye-doped nanoparticle (NP) technology which encapsulates many thousands of dye molecules inside silica matrix[51] and thus has the following advantages: (i) high intensity of the fluorescent signal, (ii) excellent photostability due to exclusion of oxygen by silica encapsulation, (iii) efficient conjugation with various biomolecules due to the silica surface which is simple to modify, and (iv) easy manufacturing process[52].

Synthesis of Dye-doped Silica Nanoparticles

There are two main methods for the synthesis of dye-doped silica nanoparticles: (a) Stöber method and (b) microemulsion method.

Stöber Method

Stöber *et al.* designed a process for synthesizing monodisperse silica nanoparticles[53]. In this process, a silica alkoxide precursor (such as tetraethyl orthosilicate, TEOS) is hydrolyzed in an ethanol and ammonium hydroxide mixture. The hydrolysis of TEOS produces silicic acid, which then undergoes a condensation process to form amorphous silica particles[54, 55]. This method can be optimized to synthesize dye-doped silica nanoparticles by covalently attaching organic fluorescent dye molecules to the silica matrix by using two-step procedure. In the first step, dye is chemically bound to an amine-containing silane agent (such as 3-aminopropyltriethoxysilane, APTS), and in the other step, APTS and TEOS are allowed to hydrolyze and co-condense in a mixture of water, ammonia, and ethanol, resulting in dye-doped silica nanoparticles[56, 57].

Microemulsion Method

This method is based on the formation of a water-in-oil reverse microemulsion system. The main reaction mixture has three main components: water, surfactant, and oil. The stabilized water nanodroplets formed in the oil solution act as small microreactors, where forms silane hydrolysis and the formation of NPs with dye trapped inside[58]. The size of NP

depends on the nature of surfactant, the hydrolysis reagent, and some other parameters, such as the reaction time, oil/water ratio, etc[59]. Organic-dye-doped silica NPs are difficult to prepare through this method because of the hydrophobic properties of the organic dye compared with the hydrophilic surface of the NPs. A modification of the protocol has been reported that increases the amount of organic dye incorporated in the particle[52]. Organic dyes are coupled to a dextran group, which is hydrophilic and can help keep the linked dye molecule within the silica. Fluorescent dyes such as tetramethylrhodamine (TMR), fluorescein, and Alexa Fluor 647 have been successfully doped into the silica NPs without leakage.

Surface Functionalization of Silica NPs

After NPs have been produced, an additional layer of linker molecules with various reactive functional groups such as amine, thiol, carboxyl or methacrylate is often attached[60]. An additional coating of the silica surface with the alkoxysilane reagent, such as carboxyethylsilanetriol is essential for introduction of carboxylic interactions. One of the most commonly accepted strategies is the attachment of avidin with net positive charge to the negatively charged NP surface through electrostatic interactions [61]. This layer provides the reaction sites for bio conjugation[62]. In addition, this step increases the repulsive forces between the particles in solution and thus improves long-term NP stability[63].

Nanoparticles Characterization

Size and Shape of Particles

The particle size is determined by the concentration of reactants (TEOS and ammonium hydroxide) for both the Stöber and microemulsion methods. It is also affected by the nature of surfactant molecules and the molar ratios of water to surfactant and co-surfactant to surfactant for the microemulsion process [59]. Transmission electron microscopy and scanning electron microscopy (SEM) are commonly used for evaluating of particle size in the vacuum state[56, 57]. Nanoparticles prepared with the microemulsion method, have spherical shape with smooth surfaces and low polydispersity, whereas Stöber nanoparticles with smaller radii (<100 nm) are less monodisperse, less spherical, and less smooth[64].

Sensitivity

In modern biomedical research and disease diagnosis, Sensitivity is a very important issue. The introduction of new fluorescent labels capable of high signal amplification is essential to address the growing need for highly sensitive bioassays. On the other hand sensitivity is crucial for monitoring rare events that are otherwise undetectable with conventional fluorophore labeling strategies. Better sensitivity can be achieved by incorporating a large number of dye molecules in a single NP system. Dye-doped silica nanoparticles exhibit extraordinary signaling strength, because of numerous dye molecules that trapped inside the matrix. For example, the effective luminescence intensity ratio of one ruthenium bipyridine (Rbpy)-doped silica nanoparticle (diameter = 60 nm) to one Rbpy dye molecule is 10^4 . Therefore, >10,000 dye molecules are presumed to be doped inside a 60-nm nanoparticle. The impressive signal of the nanoparticles can dramatically lower the analyte detection limit in biological samples[52].

Bioconjugation

Using standard covalent bioconjugation schemes, nanoparticles can act as a scaffold for the grafting of biological moieties (peptides, DNA oligonucleotides, antibodies, aptamers)[65] (Fig 1). For instance, carboxyl-modified nanoparticles have pendent carboxylic acids, making them suitable for covalent coupling of proteins and other amine-containing biomolecules via water soluble carbodiimide reagents. Disulfide-modified oligonucleotides can be immobilized onto thiol-functionalized nanoparticles by disulfide-coupling chemistry. Amine-modified nanoparticles can be coupled to a wide variety of haptens and drugs via succinimidyl esters and iso (thio) cyanates. Other approaches use electrostatic interactions between nanoparticles and charged adapter molecules or between nanoparticles and proteins modified to incorporate charged domains [66, 67].

Various studies have demonstrated that silica nanoparticles possess benign nature [69-71]. The nanoparticles exhibit little or no cytotoxicity. The biocompatibility of silica nanoparticles makes them promising for in vivo observation of cell trafficking and tumor targeting as well as for disease diagnosis and treatment.

Application of dye-doped silica nanoparticles: Dye-Doped Silica Nanoparticles in Drug Delivery

Silica NPs have been demonstrated as efficient candidate for drug delivery systems because of their intrinsic hydrophilicity, biocompatibility, and nontoxicity. In addition, these particles provide a good protection for their encapsulated drugs. With drug molecules loaded into silica NPs, surface modification of the NPs with biorecognition entities can allow specific cells or receptors in the body to be located. Upon target recognition, NPs can then release their drug payload at a rate precisely

controlled by tailoring the internal structure of the particles according to a desired diffusion profile [72]. In addition, after accumulation of the NPs in the tumor cells, irradiation of the photosensitizing drug entrapped in the NPs results in efficient generation of singlet oxygen. This singlet oxygen has a potential to cause tumor cell damage [73]. Interestingly, the discovery that mammalian cells can internalize silica NPs without cytotoxic effects, opened the door to the use of these materials as a drug delivery system. There are several reasons for the ability of dye-doped silica nanoparticles as a drug delivery system, include: (1) high surface area ($>900\text{m}^2/\text{g}$)

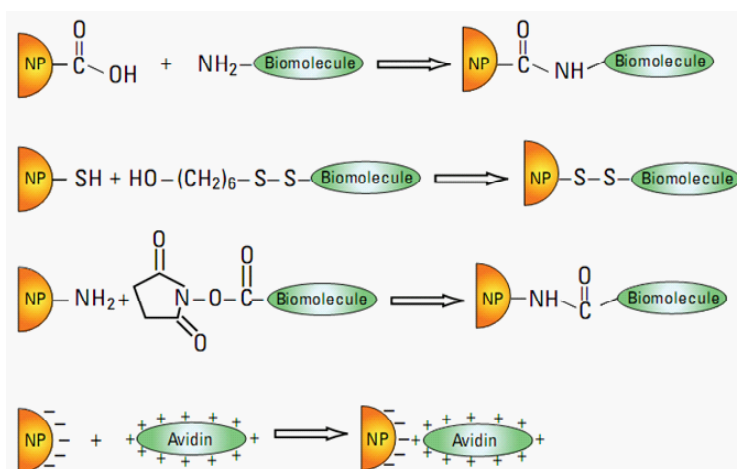


Fig. 1: Schematic procedures for attachment biomolecules to dye-doped silica nanoparticles for bioanalysis [68].

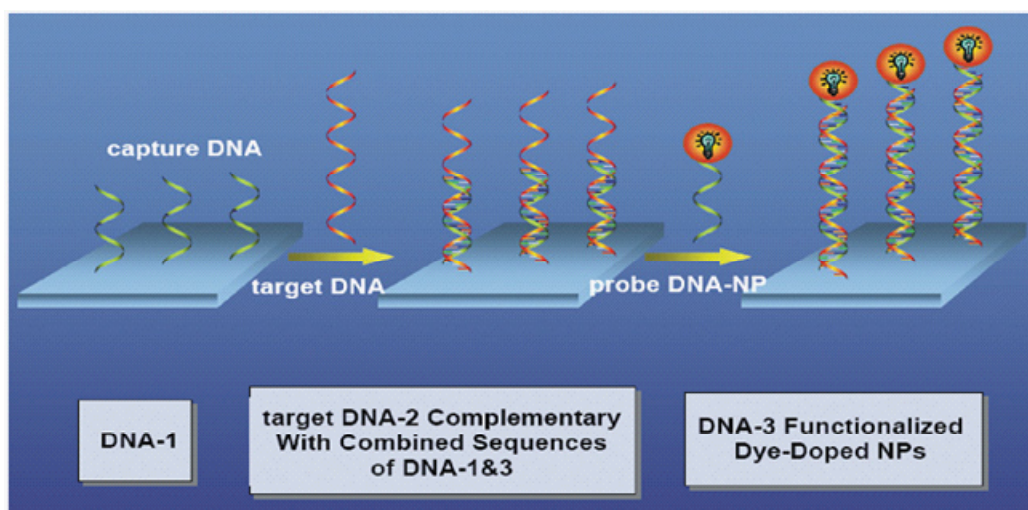


Fig. 2: Schematic diagram of a sandwich DNA assay based on dye-doped silica nanoparticles [81].

), (2) tunable pore diameter (2-20 nm) and (3) uniform hexagonal channels or cubic mesoporous structure of the silica NPs. Mesopores loaded with guest molecules were capped by inorganic NPs, or large organic molecules, via a chemically cleavable disulfide linkage to the mesoporous NP surface[74]. Physical trapping of drugs in mesoporous NP host are prevented from any premature release. Finally, the release is triggered by exposing the capped mesoporous NPs to chemical stimulation that can

cleave the disulfide linker, thereby removing the NP caps and releasing the pore-entrapped drug molecules. Indeed, this technique provides the ability to release the cargo in a controlled manner[75].

Dye-Doped Silica Nanoparticles in Gene Delivery

Silica NPs are also promising candidates for gen delivery as a nonviral vectors. The potential of cationic silica NPs was investigated for in vivo

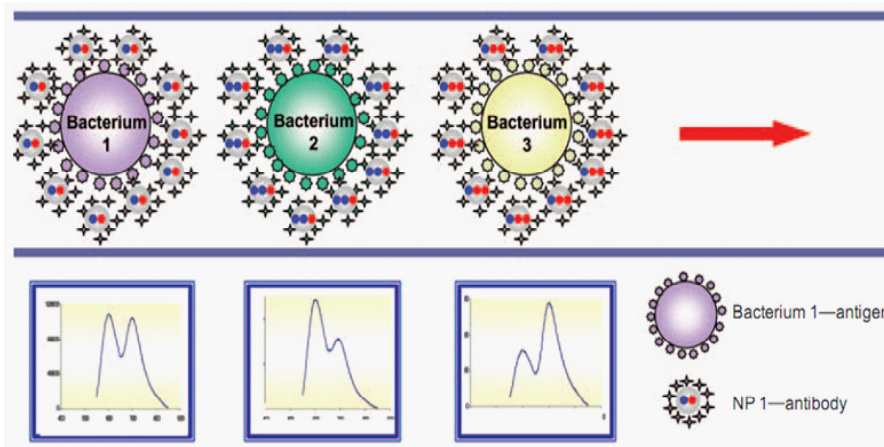


Fig 3: Schematic diagram of the dual-dye encoding system and its potential for multiplexed bacteria or cell detection within a flow system[89].

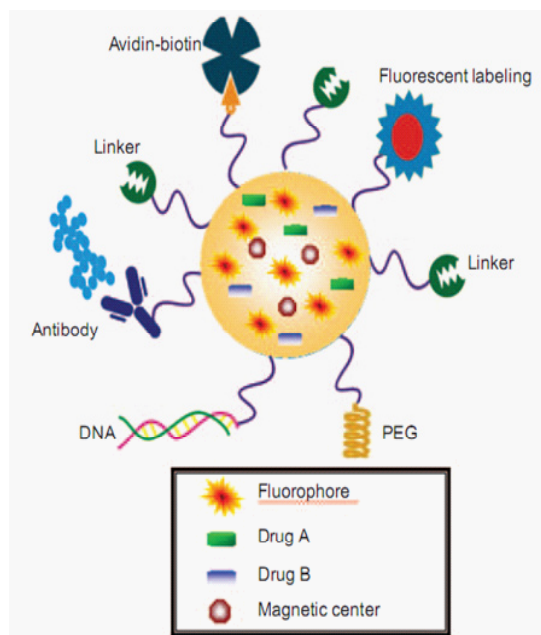


Fig. 4: Multifunctional NPs with the ability to carry one or more therapeutic agents[89].

gene transfer by Kumar *et al.* They have used these nanoparticles to transfer genes in vivo in the mouse lung. In this experiment a two-fold increase in the expression levels was found with silica particles in comparison to enhanced green fluorescent protein (EGFP) alone[76]. In another study, modified mesoporous silica NPs were used for in vivo gene delivery to the brain. It has been shown that these amino group-functionalized NPs not only bind and protect plasmid DNA from enzymatic digestion but also transfect cultured cells and deliver DNA to the nucleus[73]. In addition the process of gen delivery does not cause the tissue damage or immunological side effects[77, 78]. Therefore silica NPs have strong potential as candidates for gene transfection.

Dye-doped Silica Nanoparticles for Nucleic Acid Analysis

Dye-doped NPs can be used as labels for DNA detection by increasing in sensitivity. The scheme is based on a sandwich assay: Three different DNA species were present in the assay:

capture DNA, which was immobilized on a glass surface; a probe sequence, which was attached to TMR-doped silica NPs; and the unlabeled target sequence, which was complementary to both the capture sequence and the probe sequence through different parts of the sequence (Fig. 2). The need for labeling of the target, and the hybridization of target DNA are eliminated by using this sandwich assay, also brings one dye-doped silica NP to the surface. A large number of dye molecules can be attached on the surface for signaling. In this assay by monitoring the luminescent intensity from the surface-bound NPs, DNA target Molecules can be detected with increased sensitivity[79]. In addition, these nanoparticles can act as fluorescent labels for DNA and protein microarray technology to meet the critical demand for enhanced sensitivity. For example Cy3- and Cy5-doped silica nanoparticles have been used for two-color microarray detection in a sandwich assay format and exhibited 10 higher sensitivity than conventional cyanine dyes[80]. Indeed these nanoparticles have great potential for microarray analysis in genetic screening, proteomics, and medical diagnostics.

Dye-Doped Silica Nanoparticles for Immunoassays

Using conjugating Silica NPs to an antibody, these materials can be used as a superior signaling element, to detect cells, proteins and bacteria in an immunoassay [82, 83]. Santra *et al.* used covalent method to attach Surface-modified, Rubpy-doped silica NPs to mouse antihuman CD10 antibody. This complex was then incubated with mononuclear lymphoid target cells. After washing away the unbound NPs, target leukemia cells can be clearly detected. In comparison to control group, results of these experiment, have been shown this technique is very effective to detect leukemia cells selectively[51].

Tan *et al.* have developed Eu-doped silica NPs. In the first step, NPs were modified with streptavidin and in the next step, biotinylated antibodies were conjugated to the surface of nanoparticles. For the recognition of carcinoembryonic antigens (CEA) and hepatitis B surface antigens (HBsAg), they have used a sandwich-type time-resolved fluoroimmunoassay (TR-FIA). Results with human

sera samples correlate well with a comparative study using an established method[84].

Another experiment has been done by Houser and coworkers for the detection of choline acetyltransferase (ChAT). ChAT is a marker of cholinergic neurons in the brain. By using biotin-streptavidin conjugation, Goat anti-ChAT antibody was biotinylated and attached to streptavidin-modified NPs. This complex has a great potential for imaging of cholinergic neurons, which could be beneficial in Alzheimer's disease research[85]. Similarly, other experiments have been done by other researchers[86, 87].

Dye-doped Silica Nanoparticles for Multiplexed Bioanalysis

One of the recent developed applications of dye-doped silica nanoparticles is multiplexed bacteria detection. In this regard, two-dye encapsulated NPs have been adopted. Three different antibodies were attached to different NPs doped with varying intensity ratios of the two dyes. Each labeled NP specifically recognized and bound to the corresponding antigen-presenting bacteria. When the bacteria passed through the fluidic channel in a flow cytometer system, each kind of bacterium-NP complex exhibited the unique fluorescence signature of the attached NPs. This technique provides a selective, sensitive and rapid detection of multiple bacteria or cell (Fig 3)[88]

Multifunctional Silica Nanoparticles

Developing multifunctional NPs has made it possible to combine diagnosis and treatment procedures into a single modality. These NPs have opened promising avenues for diagnosis, treatment, and real time monitoring of biological tissues and cells. Multifunctional NPs at least, consist of three parts: featuring a core constituent material, a therapeutic or imaging payload, and biological surface modifiers, which enhance the biodistribution and tumor targeting of the NP dispersion (Fig 4). Recently, multifunctional magnetic and fluorescent NPs have been developed that consist of a thin silica shell encapsulating magnetic NPs as well as fluorescent dyes [90-92]. These NPs, can be simultaneously manipulated with an external magnetic field and characterized in situ

by optical spectroscopy[93]. For example, Kircher and coworkers, have applied near-infrared (NIR) fluorescent and magnetic NPs (32 nm in diameter) in dual labeling of brain tumors. This method was very useful for surgeons during the preoperative planning phase and surgical resection of tumors[94]. After functionalizing surface of nanoparticles with a biotargeting group, such as leutinizing hormone-releasing hormone, the NPs targeted receptor of cancer cells. Subsequent exposure to a DC magnetic field resulted in the selective destruction of the cancer cells. This study that has been done by Lu *et al.* demonstrated that multifunctional NPs are potential diagnostic and therapeutic tools for cancer and infectious diseases[95].

CONCLUSION

Fluorescent nanoparticles have great potential in cancer imaging, imaging-guided

surgery and therapy, detection of cells, proteins and bacteria in an immunoassay. Recent advances in the synthesis and pharmaceutical progress in developing efficient nanoparticles-specific agents have made revolutionary progression in molecular imaging and targeted drug delivery techniques. These particles are an ideal candidate for targeted drug and gene delivery because of their ability to protect their encapsulated content and physical properties. Moreover, the chromosomal abnormalities can be identified by Fluorescent in situ hybridization technique. In this technique, quantum dots are used for labeling of DNA. FRET is the other technique that visualizes DNA replication dynamics by using QD. Today, by developing multifunctional NPs, combining diagnosis and treatment procedures into a single modality is possible.

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