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Dye doped Nanoparticles in Biomedical Diagnostics.

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Synonyms

Dye doped nanospheres in Biomedical Diagnostics

Definition

Dye doped nanoparticles are new class of fluorescent labels. They are typically made of silica or polystyrene with tens of thousands of fluorescent dyes entrapped in the pores of its polymer matrix.

Overview and general concept

When designing devices for biomedical diagnostics, it is essential to maximize the signal to noise ratio to achieve clinically relevant sensitivity and limits of detection. Simple assay designs involve first capturing an antigen onto a surface using one antibody, then measuring the surface concentration by visualizing the captured antigen through its reaction with a second, labeled antibody. There is increasing interest in fluorescence-based array sensors (biochips) for biomedical applications. While fluorescence detection offers high sensitivity, there is generally a low level of fluorescence signal from the biochip due to a monolayer of fluorescent labels, hence the importance of enhancing the fluorescence. In antibody-based assays, the measured signal can be amplified through the replacement of molecular luminophore with doped nanoparticles (NP) (Fig. 1).

Fluorescent labels are used for a range of applications including immunosorbent assays(1), immunocytochemistry(2), flow cytometry(3) and DNA/protein microarray analysis(4). Current research in biomedical diagnostics is moving to inexpensive devices, using biochips that often have to measure biomolecules

concentrations at the picomolar level in blood sample volumes on the microliter scale, without the possibility of any user manipulation of the sample. This is exceptionally challenging to achieve in a miniature bioassay device. First, the active biomolecules (antibody or nucleic acid) must be immobilized at the appropriate surface density while maintaining its reactivity. Second, the measured signal should be maximized relative to noise and background contributions. In antibody-based assays, non-specific binding (NSB) controls the background response and often results in a detection limit that is much higher than that defined by the equilibrium constants for the binding events.

Figure 1 A cartoon illustrating the concept of fluorescence linked immunosorbent assay using the detection antibody labeled with molecular fluorophore (NIR-664 in this particular case) and 80 nm nanoparticles, doped with tens of thousands of molecular dye molecules, thus significantly increasing the brightness of the label. The TEM image shows the high monodispersity and size-uniformity of 80 nm silica nanoparticles doped with NIR-664, prepared by reverse microemulsion method. Inset with PAMAM dendrimer highlights an unique modification of the NP surface that ensures high colloidal stability and efficient bioconjugation to biomolecules of interest.

To meet these new demands, fluorescent labels with improved physical and chemical properties are required: for example, high photostability and fluorescence intensity, with a reproducible signal under a variety of chemical and biological conditions. Excellent photostability and significant reductions in photobleaching is required for applications where high intensity of prolonged excitations are applied, such as intracellular optical imaging. Dye-doped nanoparticles (typically silica or polystyrene) stand out as excellent candidates as it is possible to dope silica NPs with a large number of fluorophores, increasing the total fluorescence of the label significantly. Despite the effect of fluorescence quenching phenomena within an NP with a large amount of dye incorporated in a small volume, the goal of producing a particle with brighter fluorophores protected inside an organic matrix, thereby increasing photostability and quantum efficiency is largely successful. Owing to their intense signal, fluorescent

nanoparticles (NP) are useful as labels since they can be measured directly, without the need for any amplification step.

How to select appropriate dye doped NPs for your application?

Dye doped NPs are currently offered in wide range of sizes, with entrapped fluorophores covering most of the useful spectrum of UV/Vis radiation and with a surface modification to contain chemically reactive groups, usually –COOH and –NH_2. Most of the scientists using dye doped NPs will make their decision based on the characteristics of the instrument (e.g. excitations source and emission filters) and the type of application.

Typically, at least three parameters should be defined when selecting the appropriate dye doped NP:

a) What is the desired fluorescence property of the NP for your application?

The choice of the luminophore, particularly its excitation and emission wavelengths, obviously depends on the capacity of the instrument that is used to excite the fluorophores and collect the emitted light. However, it also depends on the application. At near infrared wavelengths there is low background interference from the fluorescence of biological molecules, solvent, and substrates. For example, whole blood has a weak absorption in the NIR region, thus reducing the need for whole-blood filtering for assays using whole blood. NIR light can also penetrate skin and tissue to several millimeters and this can enable fluorescence detection in dermatological or in-vivo diagnostic devices. When preparing dye doped NPs, it is also important to remember that not every readily available dye can be doped into the NP matrix. This will be further discussed later in this chapter.

b) What is the desired size of the NP for your application?

In case of bioassays, the answer to this question is specific to the design of the assay. While it is tempting to think that the brightness will increase with increased size of the NP, one must also consider the increased surface area and different monolayer packing arrangement of larger NP when compared to smaller ones.

Figure 2 A computer model showing the correlation between the brightness ratio and the radius and number of dye molecules entrapped inside NP.

It is not necessarily true that by increasing the diameter of NP say by order of two, the fluorescence will also increase by the same order (Fig. 2). In general, dye doped NPs in the range of 20-200 nm were successfully used in bioassays in the wider research community. As for optical imaging, the rate of uptake of nanoparticles by mammal cells depends on the particle size and shape. Number of articles emerged recently reporting that the optimum size of nanoparticles to enter the cells is around 50 nm. The cellular uptake of ~50 nm NP is typically the highest and the NPs were shown to be trapped inside vesicles in the cytoplasm, however did not enter the cell nucleus. Shape also plays an important role, with spherical nanoparticles being taken in larger numbers than rod-shaped nanoparticles. This is presumably due to the difference in curvature affecting the contact area with the cell membrane receptors.

c) What is the desired surface modification for efficient conjugation with biomolecules?

It is relatively easy to functionalize silica or polystyrene NPs with reactive groups that enable bioconjugation. However, each step in the conjugation process modifies the zeta potential of the NP, thus affecting colloidal stability. Proteins are usually immobilized on the surface of polystyrene beads by physical adsorption. It is a relatively fast method for formation of reasonably homogeneous protein layer. For better results, it is often desirable to block the non-active surface area with bovine serum albumin or similar blocking agents. In case of silica particles, the colloidal stability and the density of the active molecules on the surface can be fine-tuned by carefully executed surface chemistry. Some aspects of the surface modification strategies of dye doped silica NP will be discussed later in this chapter.

Basic Methodology

Synthesis of dye doped silica nanoparticles

One obvious advantage of using silica particles as opposed to polystyrene is the significantly higher density of fused silica (2.2 – 2.6 g/cm^3), which facilitates the manipulation and purification of samples (centrifugation at 15,000 rpm for 1-2 minutes is usually sufficient to separate the beads from supernatant). Also, silica NPs are relatively non-toxic and chemically inert. They can be prepared in range of sizes and the post synthetic surface modification can be achieved with good reproducibility.

There are two main methods for synthesis of dye-doped silica particles.

1. Sol – gel technique via Stober method, which consists of the hydrolysis of a silica alkoxide precursor (such as tetraethylorthosilicate, TEOS) in an ethanol and aqueous ammonium hydroxide mixture.
2. *Reverse microemulsion* method based on a water-in-oil reverse microemulsion system.

Both methods have certain advantages and disadvantages. For example, the former method is comparatively simple and both organic and inorganic dyes can be incorporated in the silica matrix. A modified Stober method has been used to produce highly fluorescent dye-doped core-shell silica NPs with narrow size distribution by the Wiesner group at Cornell University (NY, USA)(5). The latter technique is using stabilized water nanodroplets formed in the oil solution. Silane hydrolysis and the formation of NPs with the dye trapped inside occur inside such small microreactors. The size of the NP is determined by the nature of surfactant, the hydrolysis agent, and other parameters, such as the reaction time, oil/water ratio, etc. The resulting NPs usually show high degree of uniformity and water dispersity. One of the limitations of this method is that, in most cases, it works well with inorganic dyes, some of which have lower quantum yields compared to organic fluorophores. By both techniques, the dye molecule can be either physically adsorbed inside the pores of NP or covalently linked to its matrix. The second option is preferred, as the non-covalently attached fluorophores can

eventually diffuse or 'leach-out' into a solution over time, thus reducing the overall brightness of the NP.

Surface functionalization of dye doped NPs.

In general, the benefits of using dye doped NPs can only be realized if they are efficiently coated with biorecognition elements (in most cases antibody, oligonucleotides, etc.), have good colloidal stability and the ratio of specific to non-specific binding (NSB) is sufficiently great. The colloidal stability of NPs can be modulated by carefully executed surface modification. Sufficient separation of NPs can be achieved by increasing either steric or electrostatic repulsions between two or more adjacent NPs. Current approaches to prepare colloidal solutions with low polydispersity index are mostly based on electrostatic interactions. In polystyrene particles, the introduction of charge on the surface can be done by co-polymerization reaction of polystyrene monomer and its charged analogue. And even then, a longer term colloidal stability is guaranteed only after addition of polar surfactants into the sample. Quite often, this surfactant must be removed prior the immobilization of the antibody or DNA. On the other hand, the flexible silica chemistry provides versatile routes for surface modification. Different types of functional groups can be easily introduced onto the NP surface enabling conjugation with biomolecules of interest. The post-synthetic modification of the particle surface with charged species (e.g. phosphates, carboxylic acid, etc.) is not only aimed to reduce particle aggregation but also provide some sort of reactive handle, where the reaction with antibodies takes place. Most of the biomolecules used as molecular recognition elements are charged species (proteins, DNA, etc.) and contribute positively towards the decrease of the polydispersity index of the sample.

One of the most elegant and efficient methods to achieve good colloidal stability of silica NPs is to introduce a mixture of charged precursors on the particle surface. The first one is usually chemically inert with low pKa value, assuring that the charge is present when working at physiologically relevant pH (7.0-7.4). The second one, typically at much lower quantity provides reactive group for the

reaction with biomolecules or other cross-linking agents. In 2006, Tan and colleagues(6) proposed a way of improving the colloidal stability of silica NPs through the addition of a negatively charged, non-reactive alkoxysilane with phosphate group along with its counterpart carrying a reactive amine. Other very common groups that can be used as reactive 'handles' include carboxylic acids, isothiocyanates and epoxy group. Normally, the ratio between the non-reactive and reactive groups is relatively high (10:1) in order to maintain the suspension stability. As a consequence, the number of attachment sites on the NP surface is therefore limited. For this reason, it might be quite difficult to achieve high protein coupling ratios. Gubala(7) et al overcame this disadvantage by using multivalent linkers such as dendrimers as antibody scaffolds (Fig.3).

Figure 3 Structures of the most popular monovalent linkers and a cartoon illustrating the multivalent feature of Newkome type dendrimer, activated by dehydrating agent. (This figure was reprinted with permission of Elsevier).

Bioconjugation strategies(8)

As mentioned previously in this chapter, the most straightforward method to immobilize large biomolecules like antibodies is through physical adsorption. This method is very popular with polystyrene beads, however, it does not offer direct possibility of modulating the density of the captured protein on the NP surface. The control over the active surface area, which is the area of the NP surface covered with antibodies can be achieved by using cross-linking reagent.

There is a great choice of commercial hetero- or homo-functional linkers available; however, their effect on NP stability, aggregation, solubility and efficiency of bioconjugation was until recently poorly documented. This was striking, particularly when considering that this is the actual layer "sensed" by the molecule to be labeled.

Figure 4 Bioconjugation strategies that rely on the use of cross-linking agent. The content of $–NH_2$ (or $–NH_3^+$) groups on the surface of the NP is typically limited to ~10%. Higher amount of amines cause aggregation. The lower density of reactive amines is offset by the multivalency of dendrimers. Their effect is three fold: i) the use of dendrimers positively affects the colloidal stability of the NPs, ii) the reactivity of each amine on the surface is amplified by the number of

dendrimer's surface groups, hence significantly improving reaction efficiency with usually expensive biomolecules, iii) dendrimers, due to their size, not only serve as linkers but also as spacers between the surface of the NP and the captured recognition elements, thus maintaining the activity of antibodies, oligonucleotides, etc. The net result is more sensitive assays when compared to the monovalent linkers and also improved assay kinetics.

In a sensitive and functional bioassay, one obvious issue in the use of NPs as labels is the fraction of the coupled antibody that is in fact active or available for reaction with antigen. This fraction can be rather small, which in turn can lead to diminished sensitivity and increased non-specific binding. The minimization of NSB is essential for sensitive detection in an assay. Thus, it is clear that the strategy used to attach an antibody to NP surface is a key element that affects the activity of the bound antibody, the non-specific binding and the surface binding of particles.

Improved colloidal stability of silica NPs has been previously achieved by the addition of negatively charged non-reactive organosilanes in addition to organosilanes with functional groups available for bio-immobilisation. The number of attachment sites on the nanoparticle surface is limited in order to maintain the suspension stability of the NP system. For this reason it is difficult to achieve high protein coupling ratios. Gubala(7) and colleagues at Biomedical Diagnostics Institute have recently demonstrated some advantages of using multivalent molecules such as dendrimers as antibody coupling scaffolds as illustrated on figure 3. Dendrimers are monodisperse, nano-sized, hyperbranched 'starburst polymers', with growing numbers of terminal functional groups with increasing generation number, that have been extensively investigated for a variety of biomedical applications. By changing the generation of dendrimer, the authors were very efficiently able to control the surface area populated by biomolecules. The authors demonstrated that antibody-sensitized NPs prepared by using a multivalent linker, showed a significantly lower limit of detection and higher sensitivity than with the homo- and hetero-functional crosslinkers. The difference in performance was remarkable, particularly when compared with glutaraldehyde, one of the most common cross-linker still employed by many research laboratories. They reasoned that the multivalency of the dendrimers is

one of the most significant factors behind the increase in the detection sensitivity. Dendrimers had a positive effect on NP stability and aggregation, they were more reactive with biological materials, capable of immobilizing antibody at the appropriate surface density while maintaining its activity to the analyte of interest. Moreover, the multivalency of the dendrimer is a significant factor responsible for the improvement in the reaction yields even at lower protein concentration. This is very important, particularly when considering the cost of the bioorganic material that is used in bioconjugation reactions.

Dye doped nanoparticles and the implications on assay kinetics

A surface capture immunoassay can be considered in two ways: either incubation of antibody-sensitised particles with antigen followed by capture onto an antibody-sensitised surface of those particles that have bound antigen; or capture of antigen onto an antibody-sensitised surface followed by capture of antibody-sensitised particles onto the surface-bound antigen. In either case, the kinetics of reaction of a sensitised particle with a sensitised surface is a key element of the description of the process and hence of the assay design. The size of the particles and the fraction of the particle surface that is active for the antibody-antigen reaction are the important parameters. A simple kinetic model was developed for particle capture onto an antigen-loaded assay surface by means of an antibody-antigen reaction. This is similar to kinetic formulations treating single molecules, but also accounts for the fact that each particle has a multiplicity of antibodies present on its surface. The NPs-surface-bound antibodies can be considered as active binding sites. Depending on the coverage of such active sites on the particle, a collision of the particle with an antigen may or may not lead to reaction, with a probability proportional to the surface coverage of active sites on the particle and the surface coverage of antigen in the reaction well.

The capture reaction, of antibody-functionalised NPs onto the assay surface, can be written as:

$$P + S \leftrightarrows PS \qquad (1)$$

with forward rate constant k_{on} and reverse rate constant k_{off}. Here, S denotes an active antigen site on the assay capture surface. If the NP is bound to the surface by a single antibody-antigen interaction, then k_{off} will simply be the dissociation rate constant for the antigen-antibody complex. The association rate, k_{on}, will be determined by the probability of a reactive collision between an antibody functionalised NP and an unoccupied reactive antigen site on the assay surface. A reactive collision, leading to coupling of the NP to the assay surface, would occur when a reactive part of the NP surface (an antibody, oriented with the binding site exposed) collides with a reactive part of the assay surface (an antigen, oriented with the epitope exposed). NPs are in a continuous state of collision with the assay surface, at a rate determined by the diffusion coefficient of the NPs (hence by their radius and the viscosity of the reaction medium) and by their concentration. As the NP concentration on the assay surface builds up, particles not only occupy sites but also physically block the assay surface area, on account of their size. The probability of obtaining a successful reactive collision between particle and capture surface also depends on the fraction of the capture surface that is active for reaction. Therefore, the probability that the collision will be with an active site on the assay capture surface will be a product of the following:

- *The fraction of the total assay capture surface that is covered by accessible antigen with the binding epitope correctly exposed to facilitate antibody binding*;
- *The fraction of the total assay surface that is unblocked by bound NPs*;
- *A coverage-dependent factor that expresses the requirement that a NP requires a space whose smallest dimension is at least as large as the NP diameter, in order that the NP can fit into the space*.

Figure 5 Top – A cartoon illustrating a bioconjugation reaction between single chain fragments of C-reactive protein antibody (ScFv) and dye doped silica NP. By varying the dendrimer generation, it is possible to vary the density of ScFv fragments on the surface of NP, hence modulate the active surface area of NPs. The net outcome is increased probability of reactive collision and hence association K_{on} constant. The effect of increased active antibody loading significantly outweighed any effect of a decreased NP diffusion coefficient compared to that of a molecular dye-labelled antibody.

The considerations of reaction probability are embedded in the capture rate constant, k_{on}, which depends on both the fraction of the surface of the antigen-loaded capture plate that is in fact active for the capture reaction (that is, it depends on the surface state of the adsorbed antigen) and on the fraction of the surface of the antibody-sensitised NP that is active for reaction. It can be written

$$k_{on} = k_D \varepsilon \theta_S \theta_P \qquad (2)$$

where k_D is the diffusion-limit rate constant for NP consumption by the antigen-surface, dependent on the NP radius and the viscosity of the medium, ε is the reaction efficiency for a reactive collision, θ_S is the fraction of the capture surface area (here the antigen-loaded capture plate) that is active for the capture reaction, which in this case is dependent on the surface coverage of active adsorbed antigen on the prepared capture surface, and θ_P is the active fraction of the NP surface, in this case dependent on the surface coverage of active antibody attached to the NP. Therefore, the central objective of surface functionalisation with is to increase θ_P, which can be achieved by using dendrimer as linker.

Gubala et al have shown how the use of dendrimers as multivalent linkers effectively controls and maximizes the active fraction of the particle surface. Dendrimers have been presented as efficient coupling agents to conjugate antibodies to the surface of dye-doped silica nanoparticles. The protein binding capacity of the dendrimer-sensitised NPs increased by the same factor as the number of surface carboxylate groups on the dendrimer used. The highest generation of the dendrimers (G4.5) used in their study showed the highest surface binding rate and the highest signal in the direct binding FLISA. The effect of increased active antibody loading significantly outweighed any effect of a decreased NP diffusion coefficient compared to that of a molecular dye-labeled antibody. The practically important parameter, the ratio of the equilibrium fluorescence (offset corrected) to the non-specific offset, or signal to background ratio, increased by a factor of ~4 for the G4.5 dendrimer-conjugated NPs compared to the molecular dye-labeled antibody.

Future directions

Successful detection devices for biomedical diagnostics frequently require high sensitivity and low LOD. Moreover, a device for point-of-care diagnostics must be both inexpensive and reliable under a variety of experimental conditions. In this chapter the advantages of employing dielectric, dye doped NPs have been highlighted for the purpose of enhancing the performance of fluorescence-based assays. The objective of this chapter was to discuss the versatility of silica (or eventually polystyrene) as a host material for fluorescent dyes for many applications, specifically in the fields of nanobiotechnology and the life sciences. Silica as a host material represents an ideal scaffold for encapsulation of organic dyes in a silica matrix thus enhancing their stability and performance.

It is obvious from the growing literature on this topic that this area is still in a growth phase and that significant new developments are likely to emerge in the coming decades. Although co-doping of NPs with multiple dyes of distinguishable spectral properties has been demonstrated, the application of such barcoding NPs in multiplexed assays is expected to grow significantly. Similarly, the use of high-brightness NPs as labels in flow cytometry applications is attracting considerable interest. The versatility of silica as a host material and the feasibility to modulate the core–shell architecture provide a unique environment for innovative research towards creating integrated nanomaterials with highly fine-tuned functionality in the field of nanotechnology and beyond.

Cross-references

Biological Applications of Magnetic Nanoparticles, Biosensors, Dendrimers, Immune system interaction with synthetic particles, Nanoparticles, Nanoparticles cytotoxicity, Nanoparticles for Drug Delivery, Sol Gel Based Nanostructures

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