High Throughput Bioassays Using Nanoparticles

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Abstract: An overview of the usefulness of different nanoparticles to improve the features of high throughput separation and individual and multiplexed detection bioassays is presented. Although the development of microarray and microfluidic systems has expanded the capabilities of these high throughput assays, the combined use of NPs and these devices has provided them with new applications in drug discovery, proteomic and genomic studies, and clinical diagnosis. This article reviews the wide application field of magnetic, gold, silver, semiconductor and other nanoparticles in high throughput bioassays. Also, the versatility of the detection systems described shows that NPs are useful alternatives to fluorescent dyes, which are often used in these assays.

Keywords: Nanoparticles, high throughput bioassays, separation and detection methods.

INTRODUCTION

Nanoparticles (NPs) have emerged as new tools for the development of high throughput (HT) assays that can be easily applied to several fields, such as proteomic and genomic studies, drug discovery and diagnostic applications. NPs, like magnetic, noble metals and semiconductor (quantum dots, QDs), exhibit size-dependent physical and chemical properties that are not shown by the bulk matter. The synthesis of these NPs and their usefulness for the development of new bioassays have been widely reviewed [1-5].

NPs also offer new possibilities for the development of microarray and microfluidic devices, two of the most useful approaches to implement HTS assays involving biomolecules, such as proteins and nucleic acids. Microarrays are miniaturized analytical devices formed by rows and columns of target elements placed on a planar substrate, which are probed with the analyte or analytes [6-11]. These miniaturized platforms allow high densities and sensitivities and reduced sample volume. Microfluidic systems are devices in which fluids are manipulated in channels with dimensions of few micrometers [12-17]. These systems allow analytical processes demanding hours for their completions to be reduced to few minutes, and even to seconds, which is of great usefulness for HT assays involving biomolecular interactions.

Some of the reasons that justify the additional use of NPs in HT bioassays are as follows:

1. NPs allow miniaturization, reducing reagent and sample consumption.
2. Improvement of kinetics of the reactions involved, owing to their large surface area, giving rise to faster assays.
3. Simplification of HT separation processes using magnetic NPs.
4. Some NPs, such as noble metals and QDs, are useful alternatives to conventional fluorescent dyes, which are widely used in HT detection assays, allowing better analytical signals.

The aim of this article is to present a critical overview, which does not attempt to be exhaustive, of recent representative examples of the ability of NPs in HT separation and individual and multiplexed detection assays. Fig. (**1**) shows the different NPs used for this purpose and the wide variety of analytical techniques that can be applied, which demonstrates the versatility of NPs and their potential for the development of new HTS assays.

HIGH THROUGHPUT BIOMOLECULAR SEPARATION ASSAYS USING NANOPARTICLES

Some examples of the usefulness of NPs, mainly magnetic ones, in different HT separation assays are shown in Table **1**. Magnetic NPs have been applied in simple and fast separation processes to determine partition coefficients of drug candidates in n-octanol/water [18]. Porous silica-encapsulated magnetic NPs were preloaded with a known amount of n-octanol dispersed into a bulk aqueous phase containing the analyte and buffer. The n-octanol droplets on the NPs created an interface with the aqueous buffer phase, which significantly shortens the time required to achieve the partition equilibrium. The method requires only a very small quantity of compound, solvent and NPs and is faster than the conventional procedure. Magnetic separation and UV-visible spectrometry were used to determine the concentration of the organic compound in the aqueous phase both before and after partitioning.

Magnetic NPs have been proposed in several HT methods to accelerate protein digestion, which is an initial step in proteomic analysis. Trypsin is widely used for protein digestion as it yields tryptic peptides of an appropriate size for mass spectrometry (MS) analysis, but the process requires several hours. The use of magnetic NPs with immobilized trypsin has been proposed for the reduction of

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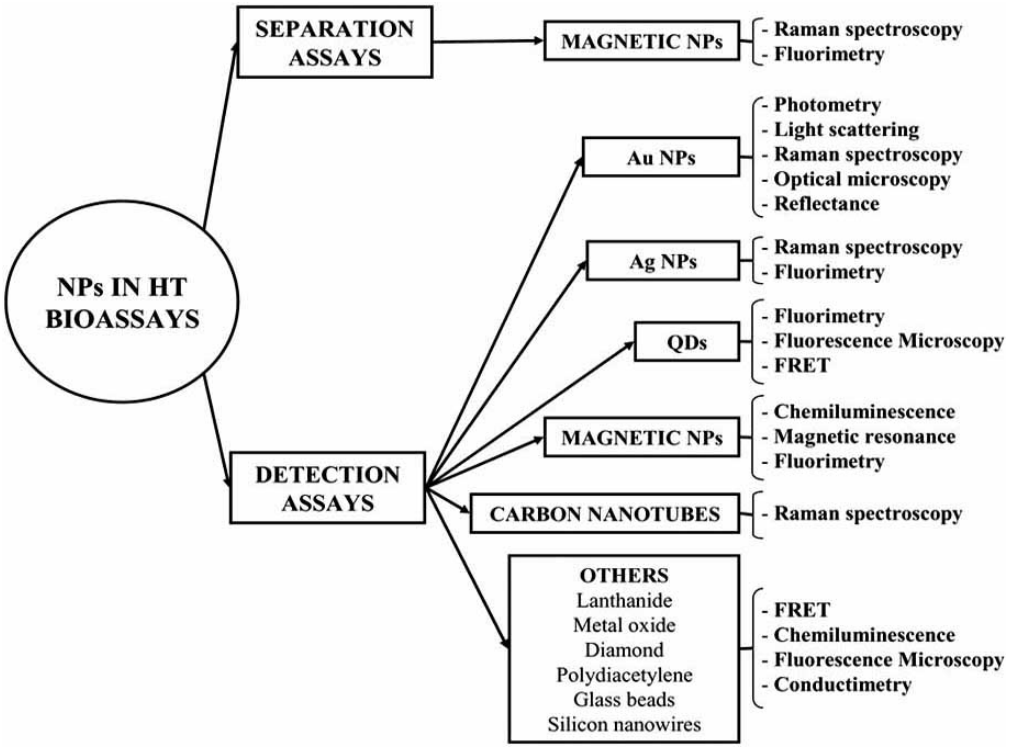

Fig. (1). Types of NPs and the different techniques to be used according to the nanomaterial.

the digestion time from hours to minutes or seconds [19-21]. Thus, a method involving microwave-assisted protein digestion has been described in which magnetic NPs are used as support for enzyme immobilization and, also, as absorbents of the microwave radiation, improving the efficiency of the process [19]. The method was applied to bovine serum albumin, myoglobin and cytochrome C, and the peptide fragments were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The enzyme was removed and reused by using an external magnetic field. A similar approach has been described to study the proteins of human lens tissue [20]. The method allowed the identification of 26 proteins compared with 11 proteins identified with traditional in-solution digestion method. Protein digestion with trypsin immobilized magnetic NPs has been also described using a microfluidic device, which has been applied to five model glycoproteins [21].

Table 1. Applications of NPs in HT Separation Bioassays

NPs	Application	References
Magnetic	Partition coefficients of drug candidates	[18]
Magnetic	Protein digestion	[19-21]
Magnetic	Phosphopeptide SPE[1]	[22-24]
ZrO_2	Phosphopeptide SPE	[25]
Magnetic	Glycopeptide SPE	[26]
Magnetic	Drug SPE	[27]
Magnetic	Antigen SPE	[28]
Diamond	Protein SPE	[29]
Polydiacetylene	Carbohydrate SPE	[30]

[1]SPE: Solid phase extraction.

Several types of magnetic NPs have been described as solid-support probes for HT separation processes using solid phase extraction (SPE). Fe(III)- [22], titanium dioxide- [23] and C_{18}- [24] functionalized magnetic NPs have been used for the isolation and enrichment of phosphopeptides, after tryptic digestion, for phosphoproteome analysis. The affinity of Fe(III) immobilized magnetic NPs to phosphopeptides has been applied to phosphoproteome analysis of the plasma membrane of mouse liver using MALDI-TOF-MS detection [22]. Titanium dioxide-coated magnetic NPs have been also used to isolate the target phosphopeptides from the sample solution using a magnetic field [23]. The identification of the peptides was carried out by introducing the NPs directly into a mass spectrometer for titania surface-assisted laser desorption/ionization mass spectrometry analysis without the need for any elution step. Tryptic digest products of β-casein and protein phosphatase inhibitor 1 were used as model samples. A similar integrated system has been described for the enrichment, detection and sequencing of phosphopeptides using C_{18}–functionalized magnetic NPs and MALDI-MS [24]. Zirconium oxide NPs have been also used to isolate phosphopeptides from the tryptic digestion of mouse liver lysate [25]. NPs with trapped phosphopeptides were isolated from the solution by centrifugation and the phosphopeptides were eluted from the NPs at pH 11.5 and detected by MALDI-MS.

Magnetic silica NPs with hydrazide groups on the surface have been used as solid supports for SPE of glycopeptides with a 96-well format [26]. These NPs have been used as an alternative to hydrazide-terminated resins, which have low capacity and difficulty to redisperse after separation from the solution phase when they are used in the cited format. However, magnetic NPs are readily separated from the

solution phase with a magnet and readily redispersed after removal of the magnetic field, which improves the development of the HT method. The captured glycopeptides were released from the magnetic NPs and analyzed by LC-MS and LC-MS-MS. Magnetic NPs have been also used as SPE probes as well as laser desorption/ionization elements for simultaneous enrichment and detection of small molecules in MALDI-TOF-MS analysis [27]. Several drugs (salicylamide, mefenamic acid, ketoprofen, flufenamic acid, sulindac and prednisolone) and triazine herbicides (simazine, simetryn, prometon, atrazine, propazine and terbutryn) were used to evaluate the general use of this approach.

Antibody-conjugated magnetic NPs have been used as an affinity probe to simultaneously preconcentrate and isolate targeted antigens from biological media [28]. The probe was combined with MALDI-MS to determine C-reactive protein and serum amyloid P component in diluted human plasma. Differential protein levels of these proteins, which are used as biomarkers, were found in samples from patients with gastric cancer and healthy individuals.

In addition to magnetic NPs, other NPs have been also described for separation purposes. Thus, diamond nanocrystallites are a special type of NPs that have shown high affinity for proteins, after being treated in strong oxidative acids. This affinity is ascribed to the interplay of electrostatic forces, hydrogen bonding and hydrophobic interactions between protein and NP surface. These NPs have been used in SPE for proteome analysis of human urine, together with MALDI-MS, without removal of the support [29]. Direct mass analysis was possible because the small size, inertness and optical transparency of the nanocrystallites. Polydiacetylene NPs have been prepared from photopolymerizable liposomes and used as glycoblotting NPs for HT protein glycomics together with MALDI-TOF MS [30]. The carbohydrates were captured by the NPs in aqueous solution, which were collected by centrifugation, and the trapped carbohydrates were released from the NPs under acidic conditions to give pure oligosaccharides. The method simplifies the purification and isolation of small amounts of sugars from biological samples, reducing the time required to less than 6 h, compared to the several days needed for conventional techniques.

HIGH THROUGHPUT BIOMOLECULAR DETECTION ASSAYS USING NANOPARTICLES

Tables **2** and **3** show the different NPs and detection systems used for the development of HT individual and multiplexed bioassays. As can be seen, most of these assays involve the use of gold (AuNPs) and silver nanoparticles

Table 2. Applications of NPs in Individual Detection Bioassays

NPs	Application	Detection System	Analytical features	References
Au	Endonuclease inhibitors	Photometry	-	[32]
Au	Tyrosine kinase inhibitors	Photometry	IC_{50} for staurosporine= 6 nM	[33]
Au	Acetylcholinesterase inhibitors	Photometry	IC_{50} for tacrine= 6.4 nM	[34]
Au	DNA binding molecules	Photometry	-	[35-37]
Au	Antimicrobial agents	Photometry	Lowest ampicillin detected = 8 μg	[38]
Au	Ovalbumin and ovomucoid	Photometry	LOD= 1 ng/ml	[39]
Au	DNA methyltransferase inhibitors	Microscopy	-	[40]
Au	IgG antibodies	Microscopy	LOD= 10 ng/ml	[41]
Au	SARS-CoV[1]	Photometry	-	[42]
Au	Kinase activity	Light scattering	LOD kemptide= 1 ng/ml	[43]
Au	DNA binders	Light scattering	-	[44]
Ag	HER2 or CD10	Raman spectroscopy	-	[45, 46]
Ag	Human IgG	Raman spectroscopy	LOD= 0.2 ng/ml	[47]
Ag	Drug-cell interaction	Fluorimetry	LOD F-WGA[2]= 1.6 pmol	[48]
Ag	Biotin-avidin system	Fluorimetry	-	[49]
QD	Viral binding inhibitors	Fluorimetry	-	[50]
QD	DNA-PKcs[3]	Fluorimetry	-	[51]
QD, Au	Avidin detection	FRET	LOD= 10 nM	[52]
QD, Au	Protein glycosylation		IC_{50}= 120 nM (22-Man-BSA)[4]	[53]
Eu(III) chelate doped NPs	Caspase-3 inhibitors	FRET	IC_{50}= 820 nM (22-Man-BSA)	[54]
Eu(III) chelate doped NPs	PSA[5]	FRET	IC_{50}= 12 nM	[55]
Magnetic	Methotrexate-dihydrofolate reductase	FRET	LOD= 0.1 ng/ml	[56]
Magnetic	Telomerase inhibitors	Chemiluminescence	-	[57]
Magnetic	SNP[6]	Magnetic resonance	-	[58]
Glass nanobeads	Rheumatoid factor	Fluorimetry	-	[59-61]
ZnO	Proteins and DNA	Chemiluminescence	LOD= 10 ng/ml	[62]
		Fluorescence microcopy	LOD= 0.02 ng/ml (anti IgG)	[63]

[1]SARS-CoV: Severe acute respiratory syndrome coronavirus, [2] F-WGA: F-WGA: fluorescein labelled wheat germ agglutinin, [3]DNA-PKcs: DNA dependent protein kinase subunit, [4]BSA with 22 manoses, [5]PSA: Prostate specific antigen, [6]SNP: Single nucleotide polymorphism.

Table 3. Applications of NPs in Multiplexed Bioassays

NPs	Application	Detection System	Analytical Features	References
QDs	Cytokines	Fluorescence microscopy	LODs= pg/ml – ng/ml	[64]
QDs	Protein expression of cancer tumor in tissue	Fluorimetry	-	[65]
QDs	Proteins in cell	Fluorescence microscopy	-	[66]
QDs, Au	Proteases	FRET	LOD caspase-3 = 20 ng/ml, LOD thrombin= 1 U/ml	[67]
QD magnetic nanobarcodes	Gene expression profiling	Fluorimetry	-	[68]
Au	Protein interactions	Raman spectroscopy	-	[69]
SWNTs	Human antibodies	Raman spectroscopy	LOD= 1 fM	[70]
Au and Ag nanowires	SNP in cytocrome P450 family	Reflectance	-	[71]
Au	IgG antibodies	Reflectance	LOD= 6.7 nM	[72]
QD nanobarcodes	Virus	Fluorimetry	-	[73]
Silicon nanowires	Genetic screening	Conductimetry	-	[74]

(AgNPs). The first applications of these NPs were reviewed several years ago [31]. However, their special and useful features have given rise to the development of new interesting applications for the detection of individual and multiple analytes, which are reviewed below. In addition to noble metal NPs, some examples of the use of other NPs for this purpose are also given.

Individual Assays

The aggregation of AuNPs, characterized by a red-to-blue color transition as the result of the red shifting of the NP surface plasmon resonance band, is a simple and fast approach for the HT detection of biomolecular interactions by colorimetric assays. Several methods have been described for the detection of enzyme inhibitors, such as endonuclease [32], tyrosine kinase [33] and acetylcholinesterase [34] inhibitors, which is a central part of the drug-development process. Cleavage enzymes like nucleases and proteases are important therapeutic targets involved in many crucial cellular events. Molecules inhibiting endonucleases, which hydrolyze the phosphodiester linkages in the nucleic acid backbone, are considered candidates for a variety of antimicrobial and antiviral drugs. Endonuclease activity has been measured using two separate batches of AuNPs functionalized with two different thiol-modified oligonucleotide strands, which are complementary to each other [32]. The hybridization of both DNA-AuNPs gives rise to the red-shift of the plasmon absorption band due to the formation of AuNP aggregates. As the enzyme degrades, the DNA-duplex interconnects, releasing the AuNPs and regenerating the red color, the system can be used for HTS of endonuclease inhibitors.

The identification of substrate and inhibitors of kinases is interesting for the development of kinase-targeting drugs since the phosphorylation of proteins by kinases plays a key role in regulating cellular processes and is involved in many diseases such as cancer, diabetes and inflammations. The aggregation of AuNPs has been applied to evaluate tyrosine kinase activity [33] using cationic substrate peptides, which act as coagulants of citrate-coated AuNPs with anionic surface charges, and phosphorylated peptides that do not coagulate AuNPs because of a net increase in the total negative charge. The method was applied to determine the activity of the tyrosine kinase c-Src and the screening of several inhibitors such as staurosporine.

The determination of acetylcholinesterase (AChE) activity and inhibitor screening have interest in Alzheimer's disease therapies. Acetylthiocholine has been used as a substrate of AChE for HTS of the enzyme inhibitors in the presence of AuNPs [34]. The method is based on the hydrolysis of acetylthiocholine into thiocholine, which induces the aggregation of AuNPs, due to the electrostatic gold-thiol interactions, and allows the measurement of the enzyme activity. Tacrine (9-amino-1,2,3,4-tetrahydro-acridine), which is an inhibitor for AChE, was selected as an example to demonstrate the usefulness of the method.

Several methods based on AuNP aggregation have been described for the detection of potential DNA-binding molecules [35-37]. Thus, an assay has been described for determining the binding affinities between these molecules and duplex DNA using networks of AuNPs interconnected with duplex DNA [35]. AuNPs were functionalized with one of two complementary thiol-modified oligonucleotide strands, obtaining the aggregates through a reversible DNA-hybridization process when the two complementary strands were combined. Aggregates of DNA-functionalized AuNP probes have been also used to determine the selectivity of a particular DNA-binding molecule for different sequences of hairpin DNA [36]. The method can be applied for potential drug candidates that work by binding DNA to form a drug/DNA complex. Also, an AuNP-modified homo-adenine DNA conjugate has been described for screening homo-adenine nucleic acid duplex binders and for identifying compounds that can selectively target the unique structure of single-stranded A-rich nucleic acids [37].

Methods for antimicrobial susceptibility assessment usually rely on the isolation of the microorganism and examination of its proliferation in the presence of different antibiotic agents. These methods are difficult to automate and to develop in an HT fashion since they require at least 24

h. A faster method, which yields results within 3 h, has been described using AuNPs and a 96-well plate [38]. The assay is based on the use of concanavalin A-induced clustering of dextran-coated AuNPs, which sense the presence of available complex carbohydrates in a bacterial suspension, and the formation of dextran-Au nanoassemblies, giving rise to the shift in the surface plasmon band of the NPs. The assay has been applied to the identification of antimicrobial agents such as ampicillin.

Resonance-enhanced absorption (REA) is an optical effect observed when noble metal nanoclusters are deposited at nanometric distance from a highly reflective mirror. An immunochip sensor based on this effect, using AuNPs, has been described for the determination of two allergenic egg white proteins, ovalbumin and ovomucoid, using a planar chip substrate and direct and sandwich immunoassay formats [39].

AuNPs, dark-field optical microscopy and digital image capture have been used in a microarray format for HTS of modulators of cleavage enzyme activity [40]. The method has been applied to detect DNA methyltransferase activity and measure the activity of drugs that inhibit this enzyme. The enzymatic cleavage activity was measured by incubating the NPs bound to the microarray surface *via* a double stranded DNA fragment, which contains enzyme specific cleavage sites, with the sample of interest. The NPs were released from the surface by the enzyme and counted with the microscope configured for dark-field illumination.

AuNPs have been also used in HT assays of biomolecular interactions in microfluidic devices, which can be applied to clinical diagnosis [41, 42]. An immunoassay for goat anti-human IgG detection is based on the deposition, observed by a microscope, of AuNPs coated with human IgG on the surface of a poly(dimethylsiloxane) (PDMS) microfluidic channel when they are in the presence of anti-human IgG [41]. In another microfluidic assay, proteins were immobilized onto AuNPs using the gold binding polypeptide (GBP) fusion method [42]. Severe acute respiratory syndrome coronavirus (SARS-CoV) envelope protein was used as model protein, which interacted with its antibody leading to changes in the absorbance and color of AuNPs.

Another simple detection system used in HT microarray assays is light scattering from metal NPs [43, 44]. AuNPs have been applied to the detection of antibody-protein binding, using proteins A and G as models, and kinase activity using the peptide substrate kemptide [43]. The avidin-biotin system was used for AuNPs attachment followed by silver deposition for signal enhancement. This detection system has been also applied in DNA microarrays to the discrimination between strong, intermediate and weak duplex and triplex DNA binders, using DNA-modified AuNPs, which can be potential anticancer agents as well as toxins [44].

Raman spectroscopy has been also proposed as a detection system in HTS methods using NPs. A method for protein detection involves the use of encapsulated surface-enhanced Raman spectroscopic tagging material composed of AgNP-embedded silica spheres and small organic molecules, such as 4-methylbenzenethiol, 2-naphthalenethiol and thiophenol, as Raman labels [45, 46]. The method has been applied to the targeting of the gens HER2 and CD10 on cellular membranes by immobilizing the antibodies on the surface of the NPs. Another immunoassay to detect human IgG involves the use of fluorescein isothiocyanate (FITC) as a Raman probe [47]. AgNPs are used to obtain Raman scattering spectra of FITC directly from silver aggregates on the bottom of a microtiter plate without displacement.

An HTS system has been described to study the interaction between drugs and cell surfaces using artificial Caco-2 cells attached to AgNPs [48]. These NPs increase the fluorescence intensity of fluorophores, such as fluorescein, due to interactions of excited state fluorophores with free electrons of the AgNPs, which polarize the metal and impose a reactive field on the fluorophores. Low power microwaves and AgNPs have been used together with fluorophores to accelerate and amplify HT protein assays [49]. The biotin-avidin system was chosen as model using a 96-well plate functionalized with AgNPs, which were coated with biotinylated-BSA and assayed with FITC-labeled avidin. It was shown that the microwaves increased the mass transport of proteins to the bottom of the wells, which increased the assay kinetics, while the AgNPs amplified the fluorescence signal.

In addition to metal NPs, other types of NPs have been described in some HT detection assays. Thus, a QD-based fluorescence assay for quantification of virus binding has been described using biotinylated human T cell leukemia virus type conjugated with streptavidin-coated QDs [50]. The assay was designed for 96-well plates and can be utilized to analyze the binding of any virus that can be biotinylated as well as for the screening of viral binding inhibitors, epitope mapping and receptor studies. QDs have been also described as labels for protein quantification in a cell lysate using a high-density microarray platform [51]. Cell lysates were spiked with DNA-dependent protein kinase catalytic subunit (DNA-PKcs) as target, which is a large protein difficult to be detected, and spotted onto nitrocellulose-coated glass slides. The results obtained were comparable to standard commercial assays based on horseradish peroxidase (HRP)-catalyzed diaminobenzidine colorimetric method.

Fluorescence resonance energy transfer (FRET) between donor and acceptor molecules is widely used in HTS systems such as those involving biomolecular interaction inhibitors. There are several examples of the usefulness of QDs and AuNPs as donor and acceptor molecules, respectively, in FRET assays. Thus, an inhibition assay for avidin detection has been proposed using streptavidin-conjugated QDs and biotinylated AuNPs, in which the presence of avidin avoids the quenching of the fluorescence of QDs by AuNPs [52]. A similar FRET system has been described in two assays to study the glycosylation degree of proteins, which is critical for the development of protein therapeutics [53, 54]. One of them involved the use of FRET between dextran-conjugated QDs and concanavalin A (conA)-conjugated AuNPs by using a miniwell plate [53]. The association between dextran and conA, which causes the fluorescence quenching of the QDs, is inhibited in the presence of a glycoprotein. The other assay, based on the use of conA-conjugated QDs and dextran-conjugated AuNPs, was carried out in a microarray system [54]. The sensitivity obtained in this instance was

lower, which can be mainly attributed to the different procedure used.

A FRET-based quenching assay for screening of caspase-3 inhibitors has been proposed using Eu(III)-chelate doped NP donors coated with streptavidin in conjunction with a dual-labeled (the N-terminal Alexa Fluor 680 fluorescent acceptor and the C-terminal BlackBerry quencher 650) caspase-3-specific peptide substrate modified with a biotinyl moiety [55]. In inhibitory conditions, the substrate remains intact and the sensitized acceptor emission is attenuated by the quencher dye. In the absence of inhibitor, the enzyme cleaves the substrate releasing the quencher, leading to emission of the fluorescent acceptor. Another FRET system has been described in a homogeneous sandwich immunoassay for prostate specific antigen (PSA) using Eu(III) chelate-doped donor NPs and near-infrared fluorescent acceptor microparticles, which were coated with two different PSA antibodies [56].

There are some examples of the use of magnetic NPs in HTS interaction assays. Thus, a chemiluminescent system has been proposed to detect the interaction between methotrexate, immobilized in magnetic NPs, and its target dihydrofolate reductase fused with the photoprotein aequorin (AQ) [57]. After the formation of the complex and magnetic separation of the NPs, the AQ-fused protein was eluted and its luminescence was measured. Magnetic NPs have been also used to develop a nanosensor, which can process hundreds of samples within tens of minutes, to measure telomerase activity in biological samples and the efficacy of different telomerase inhibitors [58]. The sensor is based on hybridization of magnetic NPs to telomerase-associated oligonucleotide repeats. The assembly formation of NPs gives rise to a change in the relaxation time of surrounding water, which can be measured by magnetic resonance relaxometers.

Magnetic NPs have been also used for single nucleotide polymorphism (SNP) detection [59-61]. A microarray system has been described using solid-phase polymerase chain reaction (PCR) on magnetic NPs [59]. A pair of allele-specific probes labeled with dual color fluorescence (Cy3, Cy5) was designed to hybridize with the PCR products amplified directly on magnetic NPs at the optimized temperature. It has been described that all steps, including PCR, hybridization, denaturation and washing can be performed in the same vessel [60]. Bacterial magnetic NPs, isolated from magnetotactic bacteria, have been used to develop a semi-automated SNP-detection processor equipped with a compact magnetic separation unit and involving Cy3 and Cy5 as labels for detection [61]. These NPs, which are more uniform in size and shape than artificial magnetic NPs, are enveloped by a phospholipid bilayer membrane, which is useful for immobilization of biomolecules such as DNA and proteins.

Glass nanobeads coated with a copolymer have been described to improve protein immobilization on microarrays [62]. Three protein microarrays for the immobilization of rabbit-IgG were compared: polymer-coated glass slides, latex microbeads immobilized at the surface of a poly(dimethylsiloxane) (PDMS) elastomer, and polymer-coated glass nanobeads entrapped at the surface of a PDMS substrate. Capture assays with peroxidase labeled antibodies and chemiluminescent detection were used, obtaining the best results in terms of protein immobilization, accessibility and analytical signal with the latter. The method was applied to the detection of rheumatoid factor in serum.

Another interesting application of NPs is the use of zinc oxide nanorods as fluorescence enhancer substrates for the detection of proteins and DNA using fluorescence microscopy [63]. The reduced dimensionality of these nanorods, when compared to zinc oxide thin films, contributed to the increased S/N ratio of biomolecular fluorescence. The fluorescence of fluorescein-conjugated antibovine IgGs, used as a model system, adsorbed on zinc oxide nanorods was visible using confocal microscopy, whereas it was no detected when these biomolecules were adsorbed on other substrates such as silicon nanorods, glass, quartz and poly(methyl methacrylate) surfaces.

Multiplex Assays

The usefulness of different NPs in multiplexed HT detection has been shown in several assays, some of them are briefly described below. The special optical features of fluorescent NPs such as QDs justify that they have been proposed as alternative labels to avoid the shortcoming of conventional fluorescent dyes, such as photobleaching and broad emission profiles, which hinder multiplexed assays. Also, the availability of nanobarcodes and nanowires has facilitated the development of these assays.

Cytokines are proteins used as cancer markers, which have the ability to stimulate or inhibit cell growth, to regulate cell differentiation, to induce cell chemotaxis and to modulate the expression of other cytokines. A multiplexed sandwich assay has been described for the detection of six cytokines using QD protein microarrays, in which capture antibodies were immobilized onto slides [64]. Two QD probes were assayed to obtain the detector antibody or tracer: 1) direct conjugation of QD to the antibody, and 2) use of streptavidin coated QDs and biotinylated antibody. The best results were obtained by the second probe. After incubation of cytokines with the capture cytokine-antibodies, a cocktail of biotinylated detector antibodies were added followed, after washing, by the addition of QD-streptavidin. After incubation and washing, the slides were imaged using fluorescence microscopy and quantified by a computerized system.

Several bioconjugated QDs have been used for quantifying protein expression of cancer tumor on tissue microarrays [65]. Tissue sections were placed on a glass microscope slide and the QD-immunostaining was performed to detect the protein markers epidermal growth factor receptor (EGFR), cytokeratin and E-cadherin. A background subtracting algorithm was used to remove tissue fluorescence signal. The excitation source was an argon-krypton laser, and the fluorescence measurements were obtained with an inverted microscope, a spectrometer and a charge-coupled device camera. Different QDs have been also used to identify and localize multiple endogenous proteins simultaneously in rat cells and mouse tissue using light and electron microscopy imaging [66]. Several examples of double and triple immunolabeling have been shown using detector antibodies linked to different QDs.

A multiplexed FRET assay for proteases, such as caspase-3 and thrombin, and their inhibitors based on the use of AuNPs as energy acceptors and QDs with different colors as energy donors, has been described [67]. QDs were arrayed directly onto NHS-derivatized hydrogel glass slide by using a robotic arrayer. After rinsing with distilled water, the slide was incubated with peptide-conjugated AuNPs, which caused the quenching of the QD fluorescence. The addition of protease alone avoids the quenching effect, whereas this effect increases in the presence of a protease inhibitor. The method can be applied to the diagnosis of protease-related diseases and screening of protease inhibitors as potential drug candidates.

A nanobarcode-based microbead random array platform has been reported for gene expression profiling in an HT and multiplexed format using QDs and magnetic separation [68]. QDs of four different sizes were mixed with a polymer and coated onto magnetic microbeads to generate the nanobarcoded beads. Gene-specific oligonucleotide probes were conjugated to the surface of each spectrally nanobarcoded bead to create a multiplexed panel and biotinylated cRNAs were generated from sample total RNA and hybridized to the gene probes on the microbeads. A fifth streptavidin QD acted as a quantification reporter by its binding to biotin on the cRNA.

There are some examples of the usefulness of Raman spectroscopy in multiplexed assays using NPs. Thus, Raman dye-functionalized AuNPs have been described for the screening of protein-small molecule and protein-protein interactions using Cy3, Cy3.5 and Cy5 as Raman dyes [69]. Biotin, digoxigenin and dinitrophenyl were chosen to study their interactions with their corresponding antibodies. Also, multiplexing capabilities of this approach were studied using mouse IgG, ubiquitin and human protein C and their respective antibodies for screening protein-protein interactions. Single-walled carbon nanotubes (SWNTs), which show sharp scattering peaks, have been proposed as multicolor Raman labels for multiplexed protein detection in an arrayed format [70]. Different antibodies were conjugated to pure ^{12}C and ^{13}C SWNT isotopes allowing multiplexed two-color SWNT Raman-based protein detection. Human antibodies against proteinase 3, a biomarker for the autoimmune disease Wegener's granulomatosis, were detected using sandwich formats.

Encoded nanowires have been used as a platform for HT single-nucleotide polymorphisms (SNP) [71]. Nanowires were obtained by electroplating alternating layers of gold and silver into the pores of an alumina template, which was removed by use of a strong base. The NPs were subsequently coated in mercaptoundecanoic acid, enabling formation of a carboxyl terminated self-assembled monolayer (SAM) on the particle surface. These NPs are intrinsically encoded by means of the different reflectivity of adjacent metal stripes. This technology was applied to analyze SNP within the cytochrome P450 family, which is an important marker in drug metabolism and cancer risk susceptibility.

There are still few examples of the use of microfluidic devices in HT multiplexed analysis. Thus, the combination of surface plasmon resonance imaging and multichannel chips has been used for the screening of four IgG antibodies against human IgG [72]. The microchannel device was fabricated with PDMS on Au-coated substrates obtained using AuNPs. Avidin was utilized as a linker layer between the surface-bound biotinylated bovine serum albumin and biotinylated anti-human IgG antibodies. The reflectance intensity increased after the immobilization of biotin anti-IgG, but the interaction of these antibodies with human IgG caused a change in this signal. Another microfluidic system has been proposed for the detection of hepatitis B, hepatitis C and human immunodeficiency virus in human serum samples using QD barcodes [73]. The process, which takes less than one hour, involves four main components: 1) QD-barcodes conjugated to pathogens, 2) electrokinetically driven microfluidics, 3) photon counting detection system, and 4) signal processing of the QD-barcode optical signals. The conjugated QD-barcodes were incubated in human serum spiked with the corresponding antibodies. Sandwich assay complexes were formed by incubation with AlexaFluor-488 organic dye conjugated to goat antimouse IgG to provide the fluorescent signal.

In addition to optical detection, the usefulness of NPs in multiplexed assays has been shown using other detection systems. For instance, conductance measurements have been proposed for genetic screening using two terminal silicon nanowire electronic devices as detectors of DNA and DNA mismatches [74]. The surfaces of these devices were modified with peptide nucleic acid receptors and applied to distinguish the wild-type DNA versus mutant oligonucleotide sequence in the cystic fibrosis transmembrane receptor gene.

CONCLUSIONS

This review article has attempted to show the usefulness of NPs in HT separation and individual and multiplexed detection bioassays by giving a series of examples. There is not doubt that the inherent chemical, optical, magnetic and electronic properties of NPs offer a great variety of options for improving the features of these assays by using different detection techniques. These new approaches, which can be achieved by combining different NPs with microarray or microfluidic systems, have opened attractive possibilities up to HT assays (Tables **2** and **3**), alternative to those traditionally preferred, which are based on conventional fluorimetry. Furthermore, the use of fluorescent NPs in fluorimetric assays avoids some shortcomings of fluorescent dyes, such as photobleaching and broad emission profiles that can hinder the development of multiplexed assays.

The application of nanotechnology to HT bioassays is a relatively recent trend and it is necessary still to overcome some limitations before its consolidation. As can be seen in Fig. (**2**), which summarizes the scope of NPs in the main fields of HT assays (drug discovery, genomics, proteomics and clinical diagnostics), they have at the moment a higher impact on the development of individual detection assays. However, it is clear that NPs have a great potential for the development of separation and multiplexed assays, which are really necessary in these bioanalytical areas.

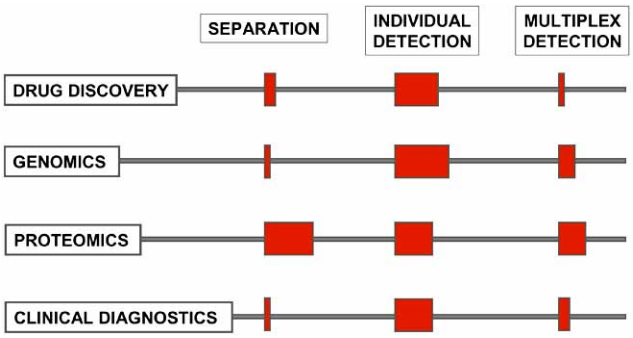

Fig. (2). Scope of NPs in HT bioassays

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