Control of Tumor Markers Using Nanotechnology

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Abstract: The relevance of tumor markers in clinical diagnosis of cancer has given rise to the development of new approaches based on the use of nanoparticles to improve the features of the immunoassays developed for their control. This article reviews the usefulness of different nanoparticles to develop direct, sandwich and competitive assays for the individual and multiplexed determination of these compounds.

Key Words: Nanoparticles, tumor markers, immunoassay.

INTRODUCTION

Tumor markers are molecules used as indicators of the presence of cancer, the response to therapy or the development of the disease. A large number of molecules have been proposed for this purpose, including glycoproteins, enzymes and hormones, which can be found in blood, urine or tissues [1-4]. An important group of tumor markers is formed by several glycoproteins such as prostate-specific antigen (PSA), α-fetoprotein (AFP), human chorionic gonadotropin (hCG) and carcinoembryonic antigen (CEA), and mucinous glycoproteins, such as cancer antigen-125 (CA 125), cancer antigen 15-3 (CA 15-3) and carbohydrate antigen 19-9 (CA 19-9).

There is wide information about the clinical interest in the control of tumor markers [1-4]. Thus, only a brief description is next given. PSA is an intracellular glycoprotein (34 kDa) used for the diagnosis of prostate cancer, which can exist in serum as free PSA and complexed with various proteinase inhibitors, such as α-1-antichymotrypsin (PSA/ACT). Elevated concentrations in plasma of AFP, which is an oncofetal protein (70 kDa), are related to hepatocellular carcinoma and terastoblastoma. Also, high concentrations of this protein in amniotic fluid may indicate severe congenital fetal defects such as spina bifida and anencephaly. hCG is a glycoprotein hormone (36,7 kDa) normally produced by the syncytiotrophoblastic cells of the placenta, increasing its level in pregnancy. The interest of hCG as tumor marker is shown in gestational trophoblastic disease and germ cell tumors. CEA (180-200 kDa) is associated to several neoplastic diseases such as colorectal, gastric, pancreatic, hepatic and cervical carcinomas. High levels in serum of CA 125 (> 200 kDa) are mainly related to epitherial ovarian cancer, while CA 15-3 (300-450 kDa) is frequently used as biomarker for breast cancer. Finally, CA 19-9 (210 kDa) is useful for clinical diagnoses of pancreatic, colorectal, gastric and hepatic carcinomas.

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These proteins are mainly determined by immunological methods in which enzymes or organic dyes, are usually chosen as labels [1]. However, there is a great interest on the use of nanotechnology to simplify and shorten these assays, which usually require several incubation, separation and washing steps. Nanoparticles (NPs) have shown their usefulness as labels, nanoscaffolds and separation media, allowing the miniaturization of the immunoassays, the increase in their sensitivity and the development of microfluidic systems. NPs exhibit new structural, electronic, optical and catalytic properties that are not shown by the bulk matter. There are several reviews describing the synthesis and properties of different NPs such as noble metals, magnetic, silica and polystyrene NPs, and quantum dots (QDs) and carbon nanotubes (CNT) [5-14]. Most of these properties have been recently used for the development of new methods, involving different analytical techniques, which facilitate the control of tumor markers in biological samples and can be considered as useful alternatives to conventional immunochemical methods. Thus, this article presents a critical overview of the different proposed assays, including a comparative study of their features and applicability and the most recent attempts to develop multiplexed methods for the simultaneous determination of these compounds.

NANOPARTICLES AND ANALYTICAL TECHNIQUES USED FOR TUMOR MARKER DETERMINATION

The interest in the use of NPs for tumor marker assays is shown through the high number of articles published in the last few years. Fig. (**1**) shows the types of NPs and the analytical techniques most commonly used for this purpose. The versatility of these NPs has allowed the use of a variety of electrochemical, mass-sensitive and optical detection systems, which have given rise to different methods that have been classified here according to the format used. As it can be seen below, direct and sandwich assays are the main formats chosen, whereas the competitive format has only been used in a few assays.

Direct Assays

Most direct assays involving NPs for tumor marker determination are based on the use of electrochemical devices,

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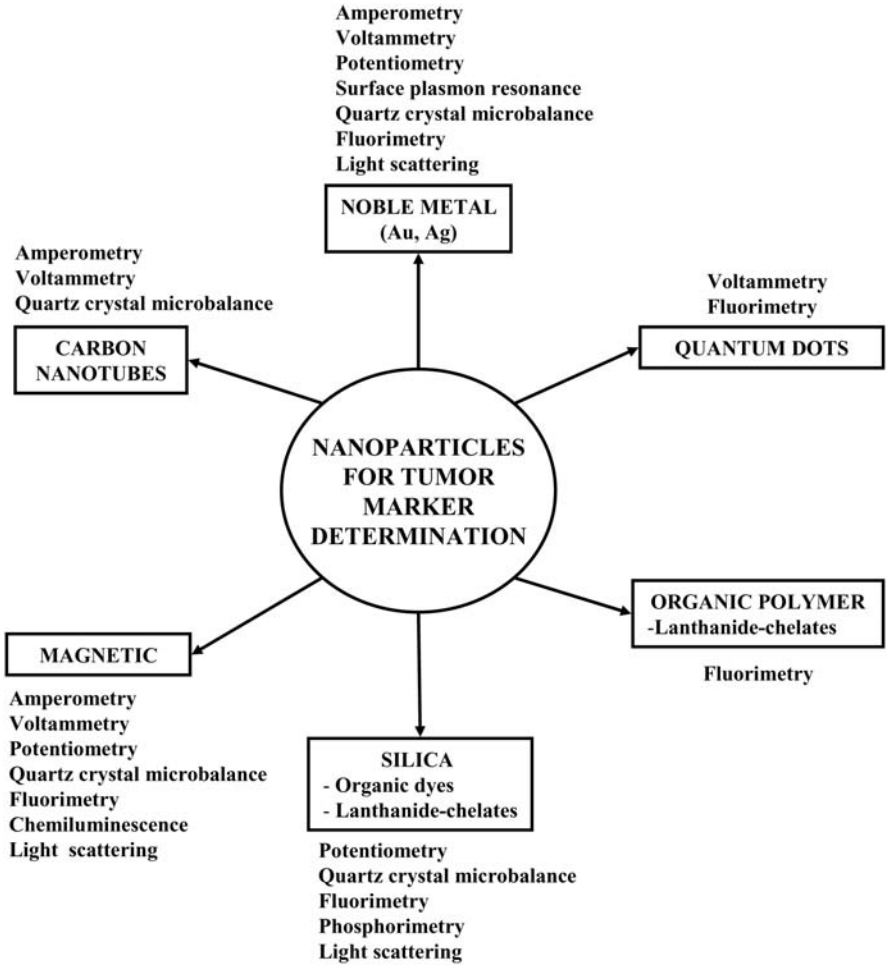

Fig. (1). Types of NPs and analytical techniques used for tumor marker determination.

prepared by immobilizing the antibodies onto the electrode surface to obtain the corresponding immunosensors. The measurement of the analyte is mainly carried out through the changes in the current intensity [15-37] and, in a lesser extension, potential [38-40], capacitance [41] or electrical resistance [42] caused by the formation of the antigen-antibody complex. The selectivity arises from the ability of the antibody to recognize its corresponding antigen. An important advantage of these direct assays is that the use of a tracer and separation and washing steps, which are usually required in conventional immunoassays, are avoided.

Fig. (**2**) shows a general scheme of these direct electrochemical assays. A crucial step for immunosensor preparation is the immobilization of the antibodies, as they must be efficiently adsorbed on the electrode surface in an oriented manner, allowing the analyte detection at low concentrations. The direct adsorption of antibodies on the electrode surface gives rise to a very low analytical signal, so that, a useful approach to increase the sensitivity of these sensors is the use of NPs, which are efficient three-dimensional nanoscaffolds. They have a high surface-to-volume area, which increases the binding capacity of the immunosensor surface. A brief description of the different NPs used for this purpose, and the procedures reported to obtain the immunoaffinity supports to modify the electrode surface, are given below.

AuNPs have been widely used to assemble antibodies for the development of direct amperometric immunosensors [15-29]. In addition to the enlargement of the sensor area, these NPs show inertness, stability to chemical and physical agents, and strong adsorption ability. Also, they facilitate electron-transfer reaction. Some of these amperometric sensors involve the use of AuNPs/TiO_2 sol-gel composite membrane that displays a porous and homogeneous architecture, in which the antibodies are encapsulated [16, 19, 25, 26]. This composite membrane has been also used with cyclic voltammetry as detection system [30, 31].

Both single- (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) have been also used in some direct assays [17, 27, 32, 41] as nanoscaffolds in electrochemical immunosensors owing to the well-defined mechanical properties of CNTs and their high ability to promote electron-transfer reactions in electrochemical measurements. It has been shown that the joint use of an AuNPs and CNTs can improve the electrochemical response. Thus, an immunosensor for AFP determination [17] is based on the use of Azure I/MWCNT composite membranes. These were prepared by adsorbing MWCNTs on a glassy carbon electrode and coating them with the Azure I dye. Subsequently, AuNPs were adsorbed by electrostatic interactions between the negatively charged AuNP and the positively charged Azure I. Antibod-

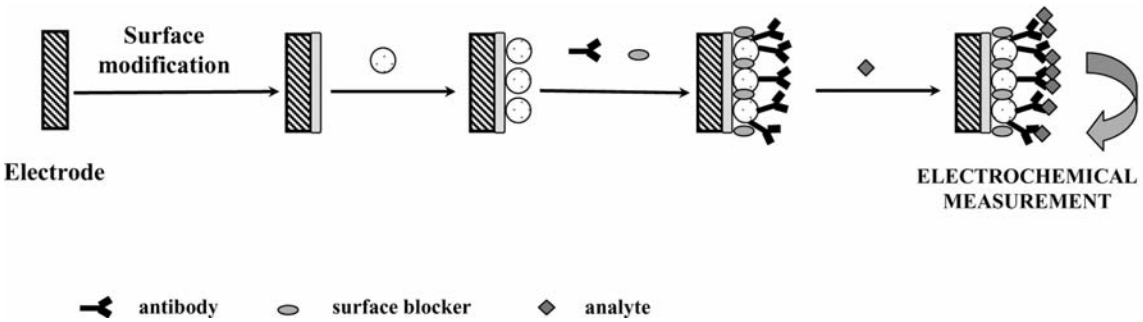

Fig. (2). General scheme of direct electrochemical immunoassays.

ies were assembled onto the surface of the AuNPs and HRP was employed to block sites against non-specific binding and to amplify the current signal. The use of HRP for these purposes has been also described in other amperometric sensors [19, 21, 22, 25, 26, 29].

Other redox dyes such as thionine [15, 26, 27, 37], prussian blue [16, 28] and methylene blue [20] have been also used as electron mediators in amperometric sensors for direct tumor marker determination. One of these immunosensors for AFP determination involves the use of a composite membrane prepared by entrapping thionine in nafion, which yields an interface containing amine groups to assemble AuNPs layer for immobilization of antibodies [15]. Thionine is immobilized by means of the electrostatic force between its positive charge and the negative charge of the sulfonate group in nafion polymer, whereas negatively charged AuNPs are immobilized by their interaction with the amine groups of thionine. Another electrochemical sensor for AFP determination is based on electrostatic interaction of CdS NPs and thionine on poly-congo red modified glass carbon electrode [37], giving rise to the formation of bilayer films with a good electrochemical activity. Cyclic voltammetry was used to obtain analytical measurements, with CdS NPs to improve the loading of antibodies and HRP to block the sites against non-specific binding and to amplify the response currents.

Differential pulse voltammetry has also shown its usefulness in the development of direct immunosensors for PSA [32], CEA [33-35], and CA 125 [36] determination, using CNTs [32], AuNPs [33, 34, 36], and magnetic NPs [34, 35]. Thus, in a flow injection assay for CEA determination in serum, the surface of a pretreated negatively charged glassy carbon electrode was modified with cationic chitosan and anionic AuNPs, previously to the immobilization of the antibodies [33]. CEA has been also determined in serum using magnetic Fe_2O_3-core/Au-shell composite NPs as affinity support for antibody adsorption [34]. These NPs were attached to a microporous polythionine modified magnetic carbon paste electrode with a permanent magnet to enhance the stability of the immobilization and avoid the leakage of the antibodies.

Potentiometric immunosensors involving direct assays by immobilizing the antibodies on the electrode surface have been described for the determination of AFP [18, 38], CEA [39] and CA 15-3 [40] in serum samples. For instance, AgNPs have been used to form a gelatine-silver film to immobilize the antibodies onto the surface of a platinum-disk electrode [38]. The film was treated with glutaraldehyde to improve the configuration. Also, the surface of a gold electrode has been modified by adsorbing the antibodies onto the surface of silica NPs, which were entrapped into a titania sol-gel composite membrane *via* a chemical vapor deposition technique [39].

Other electrochemical measurements have been used as analytical parameters for direct tumor marker determination. Thus, a direct PSA assay has been described using capacitance measurements by immobilizing the antibodies on SWNTs in a capacitor configuration [41]. After incubation with the sample, the device was washed in phosphate buffered saline solution and the capacitance was measured. Resistance measurements have been used for direct CEA quantification in serum using a flow-injection system [42]. Epoxysilane-modified core-shell magnetic Fe_3O_4/silica NPs were used as carriers for the antibodies and as electrode linkers, with the aid of an external magnet, allowing their attachment to the surface of a carbon paste electrode. The difference between resistances, before and after the formation of the immunocomplex, was used as the analytical parameter.

Quartz crystal microbalance (QCM) nanosensors are mass-sensitive transducer devices based on the measurement of resonant frequency, which decreases when the surface mass loading of the quartz crystal increases. Several direct QCM immunosensors have been described for the determination of AFP [43], CEA [44, 45], CA 15-3 [46] and CA 19-9 [47]. A critical step of the preparation of these devices is the method used to obtain the immunosensing platform in which the antibodies are immobilized. AuNPs [43, 44], magnetic $CoFe_2O_4$/silica NPs [45, 46] and CNTs [47] have been described for this purpose. CNTs have been used together with poly-L-lysine and hydroxyapatite to form hybrid NPs for the adsorption of the antibodies, avoiding the activation step of the NPs [47].

In addition to the electrochemical and mass-sensitive immunosensors above described, two interesting direct assays have been reported using resonance scattering as detection system for the determination of hCG in urine [48, 49]. Antibodies were labeled to AuNPs and the immunoreaction was monitored in the presence of polyethylene glycol, which was used as stabilizer. Another direct assay for PSA determination involves the immobilization of the antibodies on magnetic NPs and the use of Brownian relaxation time measurements in frequency domain, which depend on the hydrodynamic volume of the NPs [50]. However, the practi-

cal usefulness of this assay to the analysis of real samples has not been described.

Sandwich Assays

NPs have been used in some sandwich assays for tumor marker determination as nanoscaffolds, with the aim of increasing the amount of antibody immobilized on the sensor surface, in a similar way as the direct assays above described. This is the case of several amperometric immunosensors for PSA [51], AFP [52, 53] and CEA [54] quantification, which include the use of a tracer formed by the secondary antibody labeled with HRP. For the PSA determination in serum and prostate tissue, primary antibodies were attached onto SWCNT electrodes placed on pyrolytic graphite disks and HRP and secondary antibodies were linked to MWCNT at a high HRP/antibodies ratio [51]. Amperometric signals were developed by adding hydrogen peroxide to activate the peroxidase electrochemical cycle and measuring the current for catalytic peroxide reduction while the sensor was under a constant voltage. AFP was determined in serum by immobilizing the primary antibodies in an AuNP modified gold electrode [52]. The electrode was modified by mixed self-assembled monolayers using binary alkanethiols of different chain length or end group functionality, cysteamine and 1,6-hexanethiol. AuNPs were immobilized through two different types of interactions: covalent for the thiol and electrostatic for the amines. The amperometric detection was monitored using hydrogen peroxide and potassium iodide.

Another AFP assay involves the immobilization of the primary antibodies onto a glassy carbon electrode modified by gold nanowires and ZnO nanorods composite film [53]. The direct electron-transfer between HRP and the electrode through AuNPs gave rise to a direct electrochemical response of HRP to the hydrogen peroxide reduction. A recent assay for CEA determination in serum [54] is based on the use of a carbon fiber microelectrode covered with a well ordered antibody/protein A/AuNPs architecture. In this case, thionine-doped magnetic AuNPs were labeled on HRP-bound secondary antibody, which amplified the surface coverage of the tracer.

Most tumor marker sandwich assays are based on the measurement of a property of the NPs, which is related to the analyte concentration. A general scheme of these assays is shown in Fig. (**3**). Thus, an assay for hCG determination in urine [55] involves the use of screen-printed carbon strips in which the primary antibody is immobilized. The captured hCG was sandwiched with the secondary antibody labeled with AuNPs. These NPs were exposed to a previous oxidation process, which was subsequently followed with a reduction scan on the same surface using differential pulse voltammetry. An electrochemical immunosensor for PSA determination is based on the use of a gold electrode, in which the primary antibodies were immobilized and silica NPs colabeled with the secondary antibody and alkaline phosphatase (ALP) as tracer [56]. After the formation of the sandwich immunocomplex, ALP converted ascorbic acid 2-phosphate into ascorbic acid, which reduced silver(I) ions in the solution, leading to the deposition of silver onto the electrode surface, which was determined by linear sweep voltammetry. An interesting application of QDs, formed by CdSe and ZnS, is a method for PSA determination in serum using stripping voltammetry and an immunochromatographic strip in which primary antibodies were immobilized [57, 58]. QDs were used as labels of the secondary antibodies and detected in the test zone by measuring the dissolved cadmium with a screen-printed electrode. Since one QD contains over ten thousands of metal ions, the voltammetric signal using QD labels is greatly enhanced compared to metal ion-based labels.

The use of the optical properties of some NPs has given rise to the development of a high number of homogeneous and heterogeneous assays for tumor marker determination using the sandwich format. Thus, AuNPs exhibit bright colors due to the presence of a plasmon-absorption band that is not present in the spectrum of the bulk metal. This band is a result of the resonance of the incident photon frequency with the collective excitation of the conductive electrons of the particle. This effect is termed surface plasmon resonance (SPR) and depends on the size, shape and composition of the NPs, the distance between them and the refractive index of the environmental medium [6, 8].

Several methods based on the use of AuNPs and SPR as the detection system have been described for PSA [59-62] and hCG [63] determination, although only one of them has

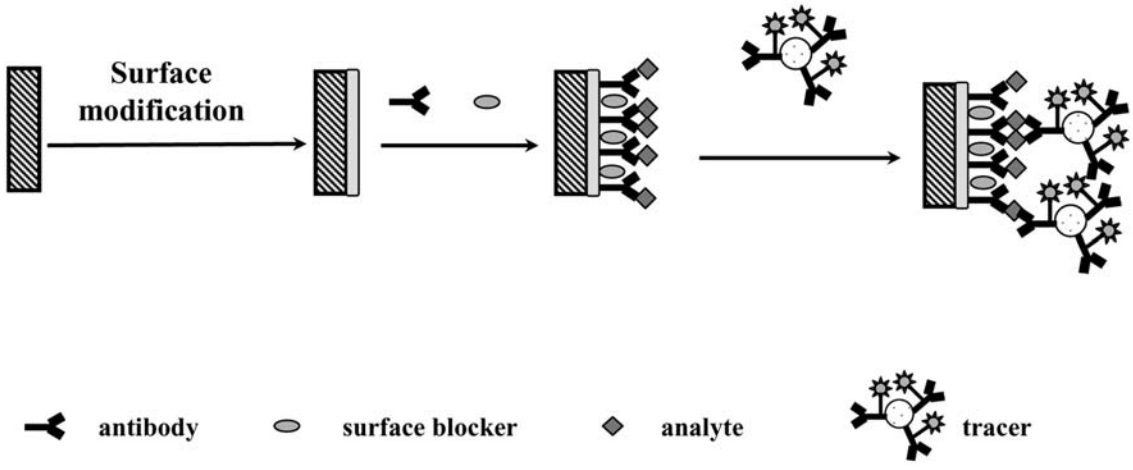

Fig. (3). Design of a sandwich immunoassay using a NP-based tracer.

shown its usefulness for the analysis of serum samples [59]. One of these assays involves the use of cystein modified recombinant protein G for the oriented immobilization of primary antibodies on a gold surface and AuNPs-conjugated secondary antibodies [60]. Another assay is based on the use of a commercially available SPR biosensor and single-domain antigen-binding fragments derived from dromedary heavy-chain antibodies (IgY) as receptor molecules, which were immobilized on a gold substrate [59]. Biotinylated mouse monoclonal antibodies and streptavidin conjugated to AuNPs were used to obtain the analytical measurement. SPR detection has been also described for the determination of PSA-α-1-antichymotrypsin (PSA/ACT) complex [61, 62]. Functionalized AuNPs were tagged with PSA/ACT complex monoclonal antibodies and with goat PSA polyclonal antibodies, and used as the probes to induce the aggregation of the NPs [61]. An amplification of the signal was obtained when AuNPs were conjugated with HRP-PSA polyclonal antibodies, using the precipitation of a substrate (3,3-diaminobenzidine) of the enzyme [62]. An immunochromatographic SPR test has been reported for the determination of hCG, which is based on the immobilization of primary antibodies in a thin zone of the chromatographic support, in which the analyte is accumulated, and the conjugation of the secondary antibodies with AuNPs [63]. Other similar immunochromatographic tests for PSA and hCG determination involve the measurement of the intense red color owing to the accumulation of the AuNPs [64-66].

Unlikely direct assays, luminescent techniques have been widely used as detection systems for tumor marker determination using the sandwich format. Fluorimetric detection has been described in an assay for AFP determination using gold and magnetic NPs [67], in which AuNPs coated with monoclonal antibodies reacted with the AFP captured by magnetic NPs also coated with monoclonal antibodies. The magnetic NPs were separated by a magnetic field and the unbound AuNPs caused the fluorescence quenching of fluorescein isothiocyanate. Another fluorimetric method for PSA determination involves the use of QDs loaded into nanobeads of 100 nm in diameter to form nanobarcodes [68]. These nanobarcodes were coated with the antibodies, using the streptavidin-biotin system. QDs have been also used, together with fluorescence microscopy, for the determination of total PSA in serum using a carbon substrate, in which the primary antibody-protein A complex was covalently bound [69]. After the reaction with the antigen, the substrate was exposed to biotinylated secondary antibodies, following of QD-streptavidin conjugates, which were captured on the substrate surface by the strong biotin-streptavidin affinity.

Time-resolved fluorimetry has been applied to PSA [70-76], AFP [77] and CEA [78] determination using several europium [70-74, 78] or terbium [75-77] chelates. These chelates were used to dope polystyrene [70-72], magnetic [73], titania [74], zirconia [76] and silica [75, 77, 78] NPs, which were conjugated to secondary antibodies to obtain the corresponding tracers. Another luminescent technique used for AFP determination in serum is solid substrate room temperature phosphorimetry, using silica NPs containing fluorescein isothiocyanate, which were prepared by sol-gel technology, as luminescent labels, and an acetyl cellulose membrane as solid support [79]. A chemiluminescent assay has been described for PSA quantification in serum immobilizing primary antibodies on nanomagnetic beads, which were bound to micro-polystyrene beads [80]. The nanomagnetic beads, produced by a magnetic bacterium, are formed by a single magnetic domain and enveloped by a lipid bilayer membrane. The magnetic separation efficiency of these NPs increased when they were assembled onto the polystyrene beads. The secondary antibodies were labeled to alkaline phosphatase and a chemiluminescent substrate of this enzyme was used for the detection.

Dynamic light scattering, also known as photon correlation spectroscopy, is based on the Brownian motion of spherical particles, which cause a Doppler shift of incident laser light. This technique has been applied in a homogeneous sandwich assay for PSA determination using spherical AuNPs conjugated with an antibody and gold nanorods conjugated with the other antibody [81]. When the two bioconjugated NPs were mixed with the antigen, the relative ratio of NP dimers, oligomers or aggregates versus individual NPs was quantitatively measured.

Surface-enhanced Raman scattering (SERS) is another technique that has shown its usefulness for tumor marker determination. It has been applied to the determination of free PSA in serum using AuNPs, which were coated with a monolayer of an intrinsically strong Raman scatterer [82].

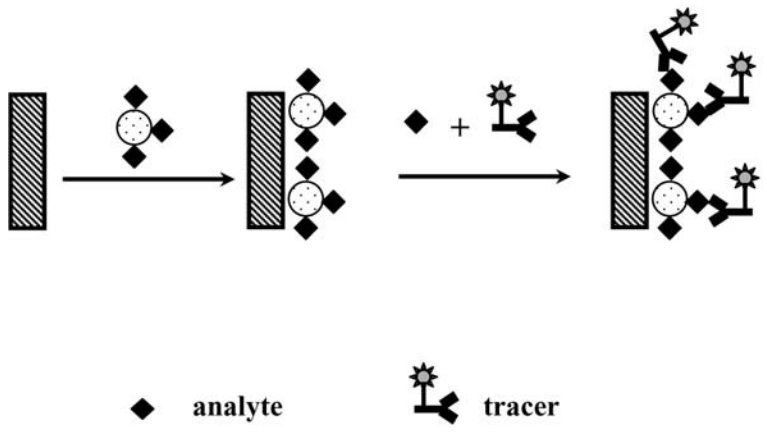

Fig. (4). Competitive assay scheme with immobilization of the antigen on the NPs.

AFP has been determined in serum using a sandwich assay performed between polyclonal antibodies functionalised Ag/silica NP-based Raman tags and monoclonal antibodies modified silica-coated magnetic NPs [83]. The magnetic NPs acted as immobilization matrix and separation tool, avoiding pre-treatment and washing steps.

Competitive Assays

AuNPs have been used as nanoscaffolds in a few competitive assays for tumor marker determination, according to the scheme shown in Fig. (**4**). Thus, several electrochemical methods have been reported for hCG [84], CEA [85] and CA 125 [86] determination in serum using HRP-antibody conjugated as tracer. These assays are based on the immobilization of the antigen in AuNPs coated on the surface of a glassy carbon [84, 86] or an indium-tin oxide [85] electrode, using trimethoxysilane [84], chitosan [85] or cellulose acetate [86] to stabilize the AuNPs in the corresponding electrode. After the interfacial competitive immunoreaction, the tracer showed enzymatic activity for the oxidation of o-phenylenediamine by hydrogen peroxide.

Another competitive assay for CA 15-3 quantification in serum involves the use of red blood cells of crucian carp and AuNPs, which were deposited on a glassy carbon electrode surface, and bound to the antibodies, using HRP-CA 15-3 as tracer [87]. The red cells of crucian carp are not easy to break up in solution. They are used in this assay as a facile redox probe for electrochemical detection and to provide the necessary binding sites with NPs. This approach has been also used with QCM, obtaining similar results.

A chemiluminescent competitive assay for PSA determination [88] involves the production of a biochip based on the electrodeposition of a gold nanostructured layer onto a screen-printed carbon microarray prior to the immobilization of PSA. Immobilized PSA was in competition with the free PSA of the sample for binding to the biotin conjugated antibody. HRP-streptavidin, luminol and hydrogen peroxide were used for the chemiluminescent detection.

Table 1. Immunoassays for PSA Determination

Detection System	Nanoparticles	Assay Format	Detection Limit	Samples	References
Differential pulse voltammetry	CNT	Direct	0.25 ng/ml	-	[32]
Capacitance	CNT	Direct	1 ng/ml	Serum	[41]
Magnetic susceptibility	Magnetic	Direct	0.1 ng/ml	-	[50]
Amperometry	CNT	Sandwich	4 pg/ml	Serum, prostate tissue	[51]
Linear sweep voltammetry	Silica	Sandwich	0.76 ng/ml	Serum	[56]
Stripping voltammetry	QDs	Sandwich	20 pg/ml	Serum	[57]
Stripping voltammetry	QDs	Sandwich	20 pg/ml	Serum	[58]
Surface plasmon resonance	Au	Sandwich	10 ng/ml	Serum	[59]
Surface plasmon resonance	Au	Sandwich	27 pg/ml	-	[60]
Surface plasmon resonance	Au	Sandwich	1 ng/ml	-	[61]
Surface plasmon resonance	Au	Sandwich	27 pg/ml	-	[62]
Color intensity image	Au	Sandwich	1 ng/ml	Urine	[64]
Color intensity image	Au	Sandwich	0.2 ng/ml	Serum	[65]
Color intensity image	Au	Sandwich	2 ng/ml	-	[66]
Fluorimetry	QDs	Sandwich	-	-	[68]
Fluorescence microscopy	QDs	Sandwich	0.25 ng/ml	Serum	[69]
Time-resolved fluorimetry	Eu(III)-chelate polystyrene	Sandwich	0.21 pg/ml	Serum	[70]
Time-resolved fluorimetry	Eu(III)-chelate polystyrene	Sandwich	0.83 pg/ml	Serum	[71]
Time-resolved fluorimetry	Eu(III)-chelate polystyrene	Sandwich	0.16 pg/ml	-	[72]
Time-resolved fluorimetry	Eu(III)-chelate magnetic	Sandwich	0.1 ng/ml	-	[73]
Time-resolved fluorimetry	Eu(III)-chelate titania	Sandwich	66 pg/ml	-	[74]
Time-resolved fluorimetry	Tb(III)-chelate silica	Sandwich	7 pg/ml	-	[75]
Time-resolved fluorimetry	Tb(III)-chelate zirconia	Sandwich	0.4 ng/ml	-	[76]
Chemiluminescence	Magnetic	Sandwich	1.48 ng/ml	Serum	[80]
Dynamic light scattering	Au	Sandwich	0.1 ng/ml	-	[81]
SERS[a]	Au	Sandwich	1 pg/ml	Serum	[82]
Chemiluminescence	Au	Competitive	5 ng/ml	-	[88]

[a] Surface enhanced Raman scattering.

ANALYTICAL METHODS

Individual Determinations

Tables **1-5** summarize some features of the methods described for tumor marker determination using NPs, showing in each instance the detection system, type of NPs, assay format, detection limit and the application to the analysis of real samples. As can be seen in Table **1**, several detection systems have been applied to the determination of PSA, using different NPs and, in most instances, the sandwich format. In general, the LODs obtained are very low, but it is worth pointing out that the lowest values are obtained using polystyrene NPs containing europium(III) β-diketone chelates and time-resolved fluorimetry. This detection system has been used in several methods, although only some of them [70, 71] have shown their usefulness for serum analysis.

Most of the methods reported for AFP determination (Table **2**) involve electrochemical measurements using AuNPs and the direct format. Although the LODs of these assays are in general very low, the lowest values have been attained in two sandwich assays involving SSRTP [79] and SERS [83], which have been applied to the analysis of serum samples.

Only AuNPs have been used in the methods described for hCG determination, as can be seen in Table **3**. In some instances, these NPs are only used as nanoscaffolds to improve the immobilization of the antibodies [19, 20, 84], although other methods involve the measurement of a property of the AuNPs, which is correlated with the analyte concentration, such as SPR [63], light scattering [48, 49] and color intensity image [64-66]. The lowest LOD reported corresponds to a sandwich assay using this last detection system [65]. The practical usefulness of these methods has been mostly shown by their application to the analysis of serum or urine samples.

Methods for CEA determination are mainly based on electrochemical measurements using AuNPs and the direct format, as Table **4** shows. The LODs reported in these methods are very similar, although the lowest value is reached using a sandwich format, silica-based europium NPs and time-resolved fluorimetry [78]. All these methods, except two [21, 28] have shown their usefulness by the analysis of serum samples. Finally, methods for the determination of the carbohydrate antigens CA 125, CA 15-3 and CA 19-9 involving NPs are not very numerous (Table **5**). Electrochemical and QCM techniques have been used as the detection systems of these methods, obtaining very similar LODs for both direct and competitive formats. All these methods have been applied to the analysis of serum samples.

Multiplexed Determinations

There is a trend to develop multiplexed detection of tumor markers using nanotechnology, although the number of methods described is still relatively low. However, the availability of multianalyte assays is advantageous to simplify testing, to increase the throughput, and to reduce overall cost. Some of these assays are described below.

A multiplexed detection system based on the use of silicon-nanowire arrays, field-effect devices and conductance measurements has been reported for the determination of PSA, PSA/ACT and CEA [89]. Monoclonal antibodies were attached to the nanowire surface and the selective binding of the tumor markers produced a conductance change in the corresponding receptor-modified silicon-nanowire device.

Table 2. Immunoassays for AFP Determination

Detection System	Nanoparticles	Assay Format	Detection Limit	Samples	References
Amperometry	Au	Direct	2.4 ng/ml	-	[15]
Amperometry	Au/TiO_2	Direct	1.0 ng/ml	Serum	[16]
Amperometry	Au/CNT	Direct	0.04 ng/ml	-	[17]
Amperometry	Au/magnetic	Direct	0.5 ng/ml	Serum	[18]
Cyclic voltammetry	Au/TiO_2	Direct	0.6 ng/ml	Serum	[30]
Cyclic voltammetry	Au/TiO_2	Direct	0.5 ng/ml	-	[31]
Cyclic voltammetry	QDs	Direct	0.12 ng/ml	-	[37]
Potentiometry	Au/magnetic	Direct	0.5 ng/ml	Serum	[18]
Potentiometry	Ag	Direct	0.8 ng/ml	Serum	[38]
QCM[a]	Au/hydroxyapatite	Direct	15.3 ng/ml	Serum	[43]
Amperometry	Au	Sandwich	5 ng/ml	Serum	[52]
Amperometry	Au/ZnO	Sandwich	0.1 ng/ml	-	[53]
Fluorimetry	Au/magnetic	Sandwich	11.9 ng/ml	Serum	[67]
Time-resolved fluorimetry	Tb(III)-chelate silica	Sandwich	0.1 ng/ml	Serum	[77]
SSRTP[b]	FITC silica	Sandwich	17 pg/ml	Serum	[79]
SERS[c]	Ag/silica, Silica/magnetic	Sandwich	11.5 pg/ml	Serum	[83]

[a]Quartz crystal microbalance; [b]Solid substrate room temperature phosphorimetry; [c]Surface-enhanced Raman scattering.

Table 3. Immunoassays for HCG Determination

Detection System	Nanoparticles	Assay Format	Detection Limit	Samples	References
Amperometry	Au/TiO_2	Direct	0.15 ng/ml	Serum	[19]
Amperometry	Au	Direct	32 pg/ml	Serum	[20]
Resonance light scattering	Au	Direct	1.5 ng/ml	Urine	[48]
Resonance light scattering	Au, Ag	Direct	91 pg/ml	Serum	[49]
Differential pulse voltammetry	Au	Sandwich	36 pg/ml	Urine	[55]
Surface plasmon resonance	Au	Sandwich	25 pg/ml	-	[63]
Color intensity image	Au	Sandwich	0.5 ng/ml	Urine	[64]
Color intensity image	Au	Sandwich	10 pg/ml	Serum	[65]
Color intensity image	Au	Sandwich	8 ng/ml	-	[66]
Amperometry	Au	Competitive	0.15 ng/ml	Serum	[84]

The practical usefulness of the nanowire sensor arrays was showed by the detection of PSA in undiluted serum samples that were desalted in a purification step. Another electrochemical method for the simultaneous detection of CEA, CA 125, CA 15-3 and CA 19-9 is based on the use of HRP-labeled antibodies modified AuNPs in biopolymer/sol-gel modified electrodes to obtain direct electrochemical responses of HRP [90]. These responses decrease when the immunocomplexes are formed, due to increasing spatial blocking and impedance.

Two microfluidic devices have been described for the simultaneous electrochemical determination of tumor markers. One of them, developed for the determination of AFP, CEA, CA 125 and CA 15-3 [91], is an arrayed immunosensor that consists of four working electrodes, each one containing a different antibody immobilized on magnet core/shell $NiFe_2O_4/SiO_2$ NPs, which were attached to the electrode surface using an external magnet. The direct assay only requires the measurement of the change in electrode potential upon binding of the antigen to the antibody. The other

Table 4. Immunoassays for CEA Determination

Detection System	Nanoparticles	Assay Format	Detection Limit	Samples	References
Amperometry	Au	Direct	0.2 ng/ml	-	[21]
Amperometry	Au	Direct	0.1 ng/ml	Serum	[22]
Amperometry	Au	Direct	0.18 ng/ml	Serum	[23]
Amperometry	Au	Direct	0.06 ng/ml	Serum	[24]
Amperometry	Au	Direct	18 pg/ml	-	[28]
Amperometry	Au/TiO_2	Direct	0.2 ng/ml	Serum	[25]
Amperometry	Au/TiO_2	Direct	0.07 ng/ml	Serum	[26]
Amperometry	Au/CNT	Direct	0.01 ng/ml	Serum	[27]
Differential pulse voltammetry	Au	Direct	0.27 ng/ml	Serum	[33]
Differential pulse voltammetry	Au/magnetic	Direct	0.3 ng/ml	Serum	[34]
Differential pulse voltammetry	Magnetic	Direct	0.5 ng/ml	Serum	[35]
Potentiometry	$Silica/TiO_2$	Direct	0.5 ng/ml	Serum	[39]
Electrochemical resistance	Silica/magnetic	Direct	0.5 ng/ml	Serum	[42]
QCM[a]	Au	Direct	1.5 ng/ml	Serum	[44]
QCM	Silica/magnetic	Direct	0.5 ng/ml	Serum	[45]
Amperometry	Au/magnetic	Sandwich	0.01 ng/ml	Serum	[54]
Time-resolved fluorimetry	Eu(III)-chelate silica	Sandwich	1.9 pg/ml	Serum	[78]
Differential pulse voltammetry	Au	Competitive	1 ng/ml	Serum	[85]

[a]Quartz crystal microbalance.

Table 5. Immunoassays for Carbohydrate Antigen (CA) Determination

Analyte	Detection System	Nanoparticles	Assay Format	Detection Limit	Samples	References
CA 125	Differential pulse voltammetry	Au	Direct	1.8 U/ml	Serum	[36]
CA 125	Differential pulse voltammetry	Au	Competitive	1.73 U/ml	Serum	[86]
CA 15-3	Potentiometry	Silica/magnetic	Direct	0.5 U/ml	Serum	[40]
CA 15-3	QCM[a]	Silica/magnetic	Direct	1.5 U/ml	Serum	[46]
CA 15-3	Amperometry	Au	Competitive	0.5 U/ml	Serum	[87]
CA 15-3	QCM	Au	Competitive	0.5 U/ml	Serum	[87]
CA 19-9	Amperometry	Au	Direct	1.37 U/ml	Serum	[29]
CA 19-9	QCM	CNT	Direct	8.3 U/ml	Serum	[47]

[a]Quartz crystal microbalance.

microfluidic device, described for the determination of PSA, AFP and CEA [92], involves the use of a microchip-based multiplex electro-immunosensing system, AuNPs and silver enhancer. The method uses a sandwich format in which capture antibodies were immobilized onto polystyrene microbeads and the secondary antibodies were conjugated to AuNPs.

An interesting biobarcode assay has been developed for the multiplexed determination of PSA, hCG and AFP in serum [93]. AuNP probes barcoded with reporter DNAs and magnetic microparticles functionalized with the capture antibodies were synthesized for this purpose. Three types of AuNP probes were prepared and each probe was cofunctionalized with reporter DNA and an antibody for each tumor marker. The analytes were exposed to the magnetic microparticles to form the immunocomplexes, followed by the bound of the AuNP probes to the resulting analyte-magnetic microparticle complex. After magnetic separation of the immunocomplexes, barcode DNAs were released, hybridized with capture DNAs printed on a chip and then identified by measuring scattered light after silver amplification. The method has been satisfactorily evaluated [94].

CONCLUSIONS

The different assays described show that NPs are really versatile tools for tumor marker determination. A large number of these assays have been focused to improve the sensitivity of electrochemical sensors increasing the amount of antibodies immobilized in the electrode surface. However, many other assays are based on the measurement of electrochemical or optical properties of the NPs. Although some technical and practical problems still need to be solved, it is important to point out that most of these assays have shown their usefulness for the analysis of real samples. The development of more multiplexed assays is desirable, since they have a great potential for clinical diagnostic applications.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support from the Spanish MCyT (Ministerio de Ciencia y Tecnología) (Grant No. CTQ2006-03263/BQU) and Junta de Andalucia (Grant No. P06-FQM-01356).

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Received: 01 December, 2008 **Revised: 14 April, 2009** **Accepted: 14 April, 2009**