Taking Advantage of Unspecific Interactions to Produce Highly Active Magnetic Nanoparticle–Antibody Conjugates

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ABSTRACT Several strategies for linking antibodies (Abs) through their Fc region in an oriented manner have been proposed at the present time. By using these strategies, the Fab region of the Ab is available for antigen molecular recognition, leading to a more efficient interaction. Most of these strategies are complex processes optimized mainly for the functionalization of surfaces or microbeads. These methodologies imply though the Ab modification through several steps of purification or the use of expensive immobilized proteins. Besides, the functionalization of magnetic nanoparticles (MNPs) turned out to be much more complex than expected due to the lack of stability of most MNPs at high ionic strength and non-neutral pH values. Therefore, there is still missing an efficient, easy and universal methodology for the immobilization of nonmodified Abs onto MNPs without involving their Fab regions during the immobilization process. Herein, we propose the functionalization of MNPs *via* a two-steps strategy that takes advantage of the ionic reversible interactions between the Ab and the MNP. These interactions make possible the orientation of the Ab on the MNP surface before being attached in an irreversible way *via* covalent bonds. Three Abs (Immunoglobulin G class) with very different isoelectric points (against peroxidase, carcinoembryonic antigen, and human chorionic gonadotropin hormone) were used to prove the general applicability of the strategy here proposed and its utility for the development of more bioactive NPs.

KEYWORDS: magnetic nanoparticles · oriented antibodies · functionalization · biosensors · molecular recognition

Over the past three decades, MNPs have found a great number of applications in fields such as drug delivery, magnetic resonance imaging (MRI), or biosensing,[1−8] to name a few. Many research lines have been devoted to establish synthetic procedures resulting in MNPs with high magnetization values, monodisperse size distribution, and high stability in a variety of environments.[9−12] In addition to synthesize "good" MNPs, it is crucial to develop procedures for their adequate biofunctionalization, which ultimately confers the appropriate features for biotechnological and biomedical applications. Therefore, a wide variety of biomolecules including aptamers, peptides, enzymes and carbohydrates have been used to provide MNPs with specificity and bioactivity.[13−17] However, among these biomolecules, Abs present themselves as very convenient candidates due to their large diversity, high specificity, and recognition efficiency.[18−21] Several strategies have been developed for the incorporation of Abs on different supports.[22−27] It must be said though that Ab functionalization of MNPs have some limitations when compared to surfaces and microbeads, such as easy aggregation with small changes of pH and/or ionic strength, polymeric shell instability, batch to batch MNPs irreproducibility, or presence of surfactants. This can be the reason why, to our knowledge, there are in the literature several contributions regarding the complexation of magnetic microbeads with Abs, but only a few for MNPs.[28−32] One of the most popular strategies entails the covalent attachment of the Ab through their most reactive amine groups. This is a simple methodology which does not involve the chemical modification of the Ab since amine groups are present in most proteins and are very reactive moieties.[33−38] However, the use of this protocol implies working at pH values below 8.0, which results in a random orientation of the Ab on the MNP surface. This is because at these pH values, the most reactive amine moieties reside in the antigen binding site of the Ab molecule, the Fab domain.[39,40] To prevent Ab random orientation, a number of strategies proposes linking the Ab in a oriented manner by alternative methods such as by using immobilized protein A[41] or protein G,[42] *via* covalent immobilization through the sugar chains of the Ab,[43,44] or by Ab site-directed biotinilation.[45−49] All

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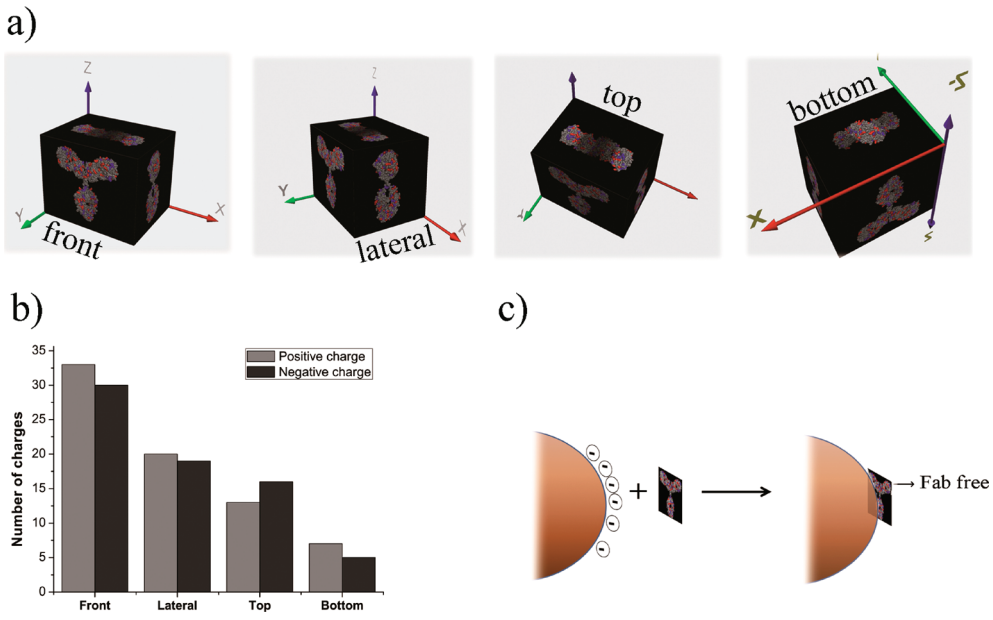

Figure 1. 3D structure of an Ab showing the positive and negative charge surface distribution. The Ab structure was taken from the Protein Data Bank (PDB) and visualized using PyMol v0.99. The PDB entry 1IGY was selected for this 3D representation. Negative and positive charges are displayed in red and blue, respectively. (a) Graphic representations of the Ab interaction planes arranged consecutively as the number of charges residues decreases, *i.e.*, front, lateral, top, and bottom views. (b) Number of charged residues per possible plane of interaction. (c) Schematic representation of the interaction of a MNP with the Ab plane of interaction (front) with the largest number of charged groups.

these strategies are based on the immobilization of the Ab through the Fc region which leaves the Fab regions available for antigen molecular recognition. Unfortunately, most of them are complex processes that imply the Ab modification through several steps of purification or the use of expensive immobilized proteins. Moreover, immobilization through oxidized oligosaccharide chains is limited to Ab bearing these chains, which is not always the case. Therefore, there is still missing an efficient, easy, and universal methodology for the immobilization of nonmodified Abs onto MNPs without involving their Fab regions during the immobilization process.

Herein, we propose the functionalization of MNPs *via* a two-step strategy which involves an initial rapid ionic adsorption of the Ab followed by a much slower Ab covalent attachment. Thus, the irreversible binding (covalent) only occurs through the region of the Ab surface where ionic adsorption took place. Summarizing, we use the unspecific reversible interactions between the Ab and the MNP in order to orient the Ab on the surface of the MNP before being attached in an irreversible way *via* covalent bonds.

RESULTS AND DISCUSSION

Since the ionic adsorption rate depends mainly on the number of charged groups present on the protein surface, ionic adsorption can be used to orient Abs. In any protein, the zone with the greater density of charged residues would have the faster adsorption rate. As a result, most of the protein molecules would be adsorbed on any support mostly through this zone in an oriented manner. Unfortunately, positive and negative charges are homogeneously distributed on any Ab surface and hence, they lack regions with either a well-defined positive or negative net charge. However, since the Ab is asymmetric, there are a number of planes of interaction which have different surface areas. The largest area, and thus, the region with the greater number of charges is the plane involving the four Ab subunits during the immobilization. Hence, our working hypothesis is that if the Ab is adsorbed through its richest negative or positive region, the antigen recognition sites will remain close to the MNP surface but they will not be involved during immobilization, preserving its antigen binding capacity (Figure 1). On the other hand, amino acid sequencing studies have shown that only small differences can be found in the protein sequence of different Abs.[50,51] These differences are all localized in the recognition site of antibodies (Fab region). The largest region of any Ab is called the constant region (Fc region) because of how well it is conserved in different Abs, even among different species. Therefore, the proposed immobilization strategy can be extended to any Ab (for an extended argument on this matter, see Supporting Information, section 2).

This functionalization strategy requires the design of MNPs containing two functional groups: (i) ionizable groups that allow the Ab ionic adsorption and (ii) reactive groups for further covalent attachment (Figure 2). Though over the past years, we have

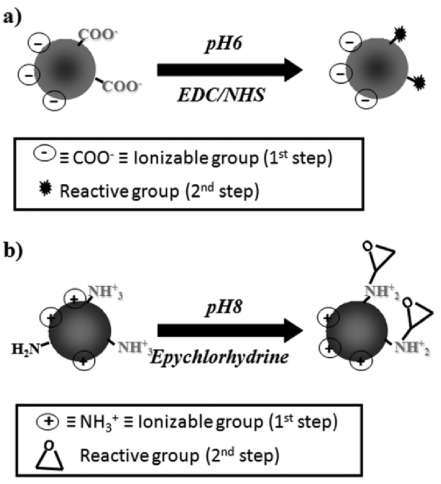

Figure 2. Novel bifunctional MNPs for binding Abs through their richest (a) positive and (b) negative charged surface regions.

extensively worked on the synthesis of bifunctional supports using agarose and epoxy-acrylic resins,[52−54] the introduction of bifunctionality on MNPs turned out to be much more complex than expected. This is mainly due to the fact that colloidal stability of MNPs depends on a delicate interaction balance among attractive (van der Waals, magnetic) and repulsion forces (electrostatic, steric). Chemical modification of the surface of MNPs is a challenging task because of their lack of stability at high ionic strength and non-neutral pH values.

As negative charges would be required on the MNP surface for binding the Ab through its richest positive charged region at the working pH, MNPs bearing carboxyl groups were selected. The partial activation of some of their carboxyl groups, with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), was optimized in order to introduce the required bifunctionality (Figure 2a). Thus, a covalent reaction can occur between the activated carboxylic groups and amino groups in the vicinity of the Ab area involved during the precedent ionic absorption. Since this strategy changes the surface charge distribution of the MNP (a negative charge is neutralized per each carboxylic group which are activated), zeta potential measurements were used to confirm the partial activation of the carboxylic groups (Supporting Information, Figure S2). This partial activation must reach a compromise between an enough number of unmodified carboxylic groups (to ensure the ionic adsorption of the Ab) and a sufficiently high amount of activated COOH groups (in order to achieve the Ab covalent attachment once it has been ionically adsorbed (Supporting Information, Table S1)).

Conversely, to bind the Ab through its richest negative charged region, MNPs containing primary amine groups were chosen. In this case, epoxy groups have been introduced by modification of the amine moieties with epichlorohydrin (Figure 2b). Epoxy groups were selected as the reactive moieties due to their ability to react with different nucleophilic groups on the Ab surface (*e.g.*, amino, hydroxyl, thiol moieties, *etc.*). The formed bonds are extremely strong bonds (secondary amino bonds, ether bonds, thioether bonds, *etc.*) with minimal chemical modification of the protein surface.[55,56] Besides, since the p*K* values of the new secondary amino groups are very similar to those of the original primary amino groups of the MNPs, this chemical modification did not affect their capacity to ionically adsorb proteins with negative net charge (anionic exchange capacity) (Supporting Information, Figure S4). To quantify the degree of introduced epoxy groups, the decrease of reactive amino groups was determined colorimetrically using Trauśs and Ellmańs reagents (Supporting Information, Table S2).

Dynamic light scattering (DLS) measurements confirmed that nonsignificant changes on the hydrodynamic diameter were introduced after both activation strategies. Therefore, MNPs did not aggregate along any of the activations strategies (Supporting Information, Table S3).

Both kinds of bifunctional MNPs validated the two-step immobilization mechanism proposed in this work. To evaluate the formation of covalent bonds after the ionic adsorption step, we took advantage of the reversible character of ionic interactions. The Ab molecules attached to the MNP only *via* ionic adsorption could be eluted away by increasing the ionic strength and/or changing the global net charge of the Ab by shifting the pH of the media. In contrast, if the Ab is covalently attached through activated carboxyl or epoxy groups of the MNP, the bond would last at any pH or salt condition.

The general structure of most Immunoglobulin G (IgG) Abs is very similar; the major difference among them is a small region at one end of the protein (the antigen recognition sites). Therefore, to optimize both immobilization strategies, antiperoxidase (anti-HRP) was selected as the working model. The anti-HRP biological activity could be determined in a straightforward way by measuring the enzymatic activity of peroxidase (HRP) that is specifically recognized by the anti-HRP. Hence, anti-HRP was incubated for different time periods with both kinds of bifunctional MNPs. In both cases, most of the attached Ab could be desorbed from the MNPs after 15 min of incubation at the suitable pH conditions. This demonstrates that ionic adsorption is very rapid and undoubtedly, faster than the irreversible covalent binding of the Ab molecules. The incubation for a longer period of time reduces the percentage of Ab that could be released from both kinds of MNPs, and ultimately, most Ab molecules were irreversible covalently attached after 2 h (Figure 3).

To confirm that the Fab region of the Ab was not involved during the immobilization, the efficiency of

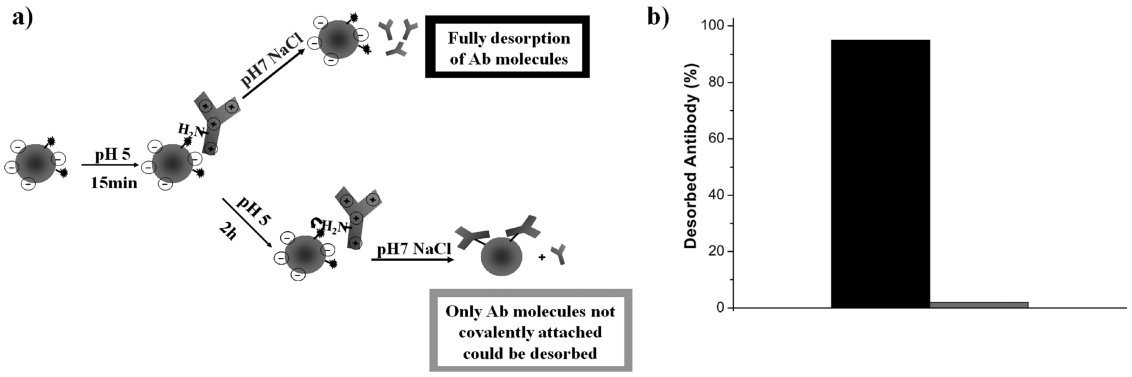

Figure 3. (a) Scheme of the two-steps immobilization mechanism proposed when using bifunctional MNPs that bind the Ab through its richest positive charged area. (b) Results obtained using this strategy that confirms the mechanism proposed. Similar results were obtained with amino-epoxy MNPs. In black desorption after 15 min; in gray desorption after 2 h.

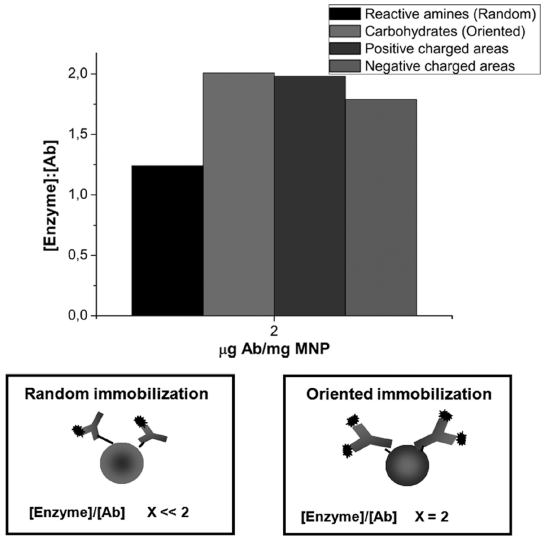

Figure 4. Capacity to capture HRP of the anti-HRP anchored to MNPs by different orientations. The protein content of all anti-HRP-functionalized MNPs was similar (2 μg Ab per mg MNP).

the Abs covalently attached to MNPs able to recognize HRP was evaluated (Figure 4). MNPs functionalized with anti-HRP immobilized through their more reactive amine groups or through their sugar chains were also prepared as control for random or oriented immobilization, respectively. All the Ab-MNP preparations were incubated with an excess of HRP. After several washings, the amount of HRP bound was quantified spectrophotometrically by determining the biological activity following the oxidation of the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). MNPs without Ab or with an antibody that did not bind HRP (anti-hCG) but blocked with bovine serum albumin (BSA) were used as blanks of HRP unspecific binding to the MNPs surface. Provided that in none of the cases HRP unspecific binding to the MNPs was observed, the enzymatic activity of each Ab-MNPs preparation can be used directly to calculate the average of HRP molecules joined by a molecule of antibody in each immobilization strategy (more details in Supporting Information: section 3.1).

If the Fab regions are sufficiently exposed to the medium, the molar ratio of antigen to Ab (HRP to anti-HRP) should be two since IgG Abs have two antigen binding sites. In the case of random immobilization, a decrease of this ratio was observed (close to 1) (Figure 4). This result confirms that the Fab zones of many Ab molecules are involved during the immobilization strongly reducing their antigen recognition efficiency. As expected, HRP to anti-HRP ratio was close to two when the Ab was immobilized by its sugar moieties. In the case of Abs immobilized by the proposed two-step strategy, both positive and negative MNPs, the biological activity of the Abs was close to the expected value (>90% efficiency in both cases). In each immobilization strategy, the final anti-HRP content per m^2 was the same, since the size of both aminated and carboxylated MNPs was 200 nm and the amount of bound Ab was similar in all the cases. Therefore, the differences observed in the HRP to anti-HRP ratio must be a consequence of a different orientation of the Ab on the MNP surface. The use of this new two-step immobilization strategy allows to obtain MNPs functionalized with fully active Abs.

As already mentioned, the general structure of all IgG Abs is very similar. In that sense, the developed methodology must be applicable to most of the Abs. To favor the initial ionic absorption step, the incubation pH must be adjusted depending on the Ab pI. As both strategies gave similar results, a preferential use of any of them would be only motivated by the colloidal stability of the MNPs at the pH where Ab ionic adsorption is most favorable.

Hereby to show the most general feasibility, antibodies against human chorionic gonadotropin hormone (anti-hCG) and carcinoembryonic antigen (anti-CEA) were bound through its richest positive charged region to partially activated carboxylated MNPs. Anti-hCG and anti-CEA have significant differences in their pIs (6.55−6.85 and 7.35−8.15, respectively) in comparison with anti-HRP (pI = 5.2−6.55) (Supporting Information, Figure S6). As in the case of anti-HRP, the

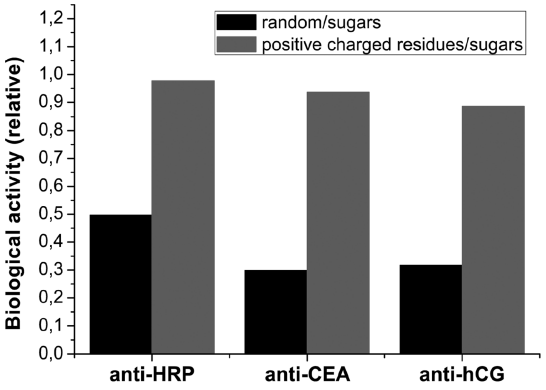

Figure 5. Relative biological activities of MNPs functionalized with anti-HRP, anti-CEA, and anti-hCG using random orientation and our two-step strategy. Results are normalized to the biological activity of MNPs functionalized with Abs through its sugar moieties (positive control).

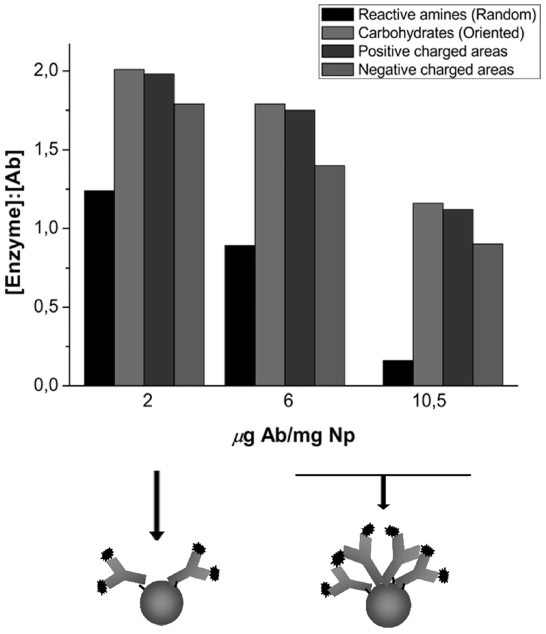

Figure 6. Influence of antibody density in the antigen binding capacity of anti-HRP functionalized MNPs.

antigen binding capacity was reduced dramatically when the Ab was immobilized in a random manner. The biological activity of the Ab immobilized using the proposed strategy was again close to the value obtained when they were linked through their sugar moieties (Figure 5). Thus, the general applicability of the strategy here proposed was demonstrated by choosing Abs with pIs covering a broad range of pH values (5.20−8.15).

Another key point to be considered in the optimization process is the surface density of Abs on the MNPs. As shown in Figure 6, an increased Ab density reduced their antigen binding capacity as the spatial accessibility of the antigen is reduced due steric hindrance between Abs in close proximity. Even when Abs were linked in an oriented way, a strong reduction of their activity was observed when MNPs were overloaded.

The reduction of antigen binding capacity due to steric hindrance increases dramatically with the complexity of the detection method. In Figure 5, the differences observed between oriented and random methodologies are higher when using an indirect antigen detection method, that is, anti-hCG and anti-CEA (Supporting Information, sections 3.1.2 and 3.1.3). These differences cannot be attributed to different Ab loadings since the amount of Ab attached to the MNPs was the same in all cases (6 μg Ab per mg of MNP).

CONCLUSIONS

The activity of Abs immobilized on MNPs is a balance of two factors: density and orientation. The results shown here demonstrate that maximizing the amount of Ab is not as advantageous as frequently assumed. Moreover, we have shown that by controlling unspecific ionic adsorption, it is possible to covalently attach the Ab in an oriented way, preserving its biological activity. The developed methodology is applicable to most Abs as long as the pH of incubation is adjusted depending on the Ab pI. Moreover, this conjugation strategy can also apply to other proteins (*i.e.*, enzymes, protein receptors, *etc.*), and it can be easily optimized for the use of another kind of nanoparticles or nanostructured materials (for more details see section 4 in the Supporting Information). For short-term applications, MNPs functionalized with anti-hCG and anti-CEA have interesting diagnostic applications. The antigen hCG is not only a reliable marker of pregnancy but also a marker of some types of testicular and ovarian cancers (germ cell tumors) and gestational trophoblastic disease.[57−59] On the other hand, CEA is the preferred tumor marker to help predict outlook in patients with colorectal cancer.[60,61] The proposed methodology can be very suitable for more sensitive, selective, and bioactive nanodevices.

METHODS

Synthesis of Bifunctional Magnetic Nanoparticles. *Partially Carboxylic Activated Magnetic Nanoparticles.* A 10 mg portion of carboxylated magnetic nanoparticles (1601/08, 0302/08, 1505/08, 1711/08, 0212/08, 1001/09, 1402/09, 0605/09, 0906/09, 3008/09 from Chemicell) was washed several times with 1 mL of 10 mM MES pH 6.1. Then the NPs were incubated for 30 min at 37 °C with 1 mL of 10 mM MES pH 6.1 containing 5 μmols of EDC and 7.5 μmols of NHS. After that, they were washed three times in the same buffer and used immediately.

Amino-epoxy Activated Magnetic Nanoparticles. A 10 mg portion of aminated magnetic nanoparticles (1901/08, 1602/08, 2804/08, 1411/08, 1711/08 from Chemicell) was washed several

times with 1 mL of 10 mM sodium bicarbonate pH 8.5. Then, the NPs were incubated 16 h at 25 °C in 25 mL of 10 mM sodium bicarbonate pH 12.0 containing 10% acetone and 0.3% epyclorohydrin. Afterward, the activated supports were washed several times with 10 mM bicarbonate pH 8.0 and stored in this buffer at 4 °C until used.

General Procedures for Characterization of the Chemical NPs Functionalization. To verify the partial activation of carboxylated MNPs, zeta-potential measurements were carried out in MNPs activated with EDC/NHS. In the case of aminated MNPs, their activation with epoxy groups was characterized by determining the decrease of reactive amine groups colorimetrically using Trauts and Ellmans reagents. More details on the characterization tools used can be found in the Supporting Information, section 1.2.

Light scattering and zeta-potential measurements were performed on a Brookhaven Zeta PALS instrument at 25 °C. Each sample was measured three times, combining 10 runs per measurement.

Covalent Immobilization of the Antibody on Magnetic Nanoparticles. *Immobilization via the More Reactive Amine Groups of the Antibody.* A 10 mg portion of partially carboxyl activated nanoparticles was incubated with 1 mL of 10 mM MES pH 7.0 300 mM NaCl with 100 μg/mL of antibody solution during 2 h at 37 °C. Then, the remaining carboxyl activated groups were blocked at 25 °C for 16 h with 1 mL BSA 1% in 10 mM MES pH 6.1. Finally, they were washed several times with 10 mM MES pH 6.1 and stored at 4 °C until used.

Immobilization via the Carbohydrates Moieties of the Antibody. One milliliter of an antibody solution 1 mg/mL in 10 mM sodium phosphate pH 7.0 was incubated with 100 μL of 0.1 M $NaIO_4$ (prepared in water) during 2 h at 4 °C and preserved from light. Then, the oxidized antibody was purified by Sephadex G-25 gel filtration column with 10 mM sodium phosphate pH 8.0. Aliquots of 10 mg of aminated nanoparticles were incubated during 2 h at 37 °C with 1 mL of a 100 μg/mL solution of oxidized antibody in 10 mM sodium phosphate pH 8.0. Then, $NaCNBH_3$ was added in order to have a final concentration of 0.25 M. After 30 min at 37 °C, the functionalized nanoparticles were washed three times with 10 mM sodium phosphate pH 8.0. Finally, the remaining amine groups of the nanoparticles were blocked by incubation with 1 mL BSA 1% in 10 mM MES pH 6.1 for 16 h at 25 °C and were stored at 4 °C until used.

Immobilization via the Positive-Charged Richest Zone of the Antibody. A 10 mg portion of partially carboxyl activated nanoparticles was incubated during 2 h at 37 °C with 1 mL of 10 mM MES pH 5 or pH 6 with 100 μg/mL of anti-HRP or anti-hCG and anti-CEA respectively. Then, the remaining carboxyl activated groups were blocked at 25 °C for 16 h with 1 mL BSA 1% in 10 mM MES pH 6.1. Finally, they were washed several times with 10 mM MES pH 6.1 and stored at 4 °C until used.

Immobilization via the Negative-Charged Richest Zone of the Antibody. A 10 mg portion of amine epoxy-activated magnetic nanoparticles was incubated with 1 mL of 10 mM sodium phosphate pH 8.0 with 100 μg/mL antibody solution for 24 h at 37 °C. The remaining activated groups were blocked at 25 °C for 16 h with 1 mL BSA 1% in 10 mM sodium phosphate pH 8.0. Then, they were washed several times with 10 mM sodium phosphate pH 8.0, and stored at 4 °C until used.

Determination of the Immobilized Antibody. *Determination of the Amount of Immobilized Antibody.* Samples of supernatants before and after every step in the immobilization process were withdrawn and measured using Bradford assay.[62] A reference solution was prepared with exactly the initial antibody concentration and media conditions (pH, ionic strength) and BSA was used as protein standard. Therefore, the decrease in the supernatant concentration can be directly correlated to the amount of the antibody immobilized (μg Ab/mg MNPs). NATIVE-PAGE (polyacrylamide) electrophoresis was also used to reconfirm the obtained results (more details in Supporting Information, section 2.1).

Determination of the Biological Activity of the anti-HRP-MNPs Preparations. A 10 mg portion of nanoparticles was incubated with 1 mL of 1 mg/mL of peroxidase (HRP) in 10 mM MES pH 7.0 300 mM NaCl during 30 min. After that, the NPs were washed several times with 10 mM MES pH 7.0 300 mM NaCl. Then, 10 μL of this solution were added on 1 mL of a solution ABTS-H_2O_2 (100:1, v/v) in 50 mM sodium phosphate buffer, pH 6.0. The increase in the absorbance at 414 nm is measured. One unit of enzyme (IU) was defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of substrate per minute under the specified conditions (more details in Supporting Information, section 3.1).

Determination of the Biological Activity of the anti-hCG-MNPs Preparations. Aliquots of 10 mg of all Ab-MNPs preparations were incubated with 1 mL of 1000 mUI/mL of hCG in 10 mM sodium phosphate pH 7.0 during 30 min. After that, they were extensively washed with 10 mM sodium phosphate pH 7.0. Then they were incubated with 1 mL of 0.5 mg/mL anti-hCG clon 5016 for 30 min in 10 mM MES pH 7.0 300 mM NaCl. After extensive washings with 10 mM MES pH 7.0 300 mM NaCl, the samples were incubated with 1 mL of 0.25 mg/mL polyclonal goat Antirabbit/HRP in 10 mM sodium phosphate pH 7.0 during another 30 additional minutes. Again, after extensively washing, the amount of bound polyclonal goat Antirabbit/HRP was quantified spectrophotometrically by determining the HRP activity present on each functionalized MNP sample following the oxidation of ABTS. The enzyme activity was measured under identical conditions already described (more details in Supporting Information, section 3.2).

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Supporting Information Available: Details on the characterization of MNPs biofunctionality (zeta potential, DLS, determination of the reactive groups of the MNPs) and on the validation of the proposed immobilization methodology (Bradford assay, electrophoresis, and biological activity). This material is available free of charge *via* Internet at http://pubs.acs.org

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