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Useful Oriented Immobilization Of Antibodies On Chimeric Magnetic Particles: Direct Correlation of Biomacromolecule Orientation with Biological Activity By AFM Studies.

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Abstract

The preparation and performance of a suitable chimeric biosensor based on antibodies (Abs) immobilized on lipase-coated magnetic particles by means of a standing orienting strategy are presented. This novel system is based on hydrophobic magnetic particles coated with modified lipase molecules able to orient and further immobilize different Abs in a covalent way without any previous site-selective chemical modification of biomacromolecules. Different key parameters attending the process were studied and optimized. The optimal preparation was performed using a controlled loading (1 nmol Ab x g^{-1} chimeric support) at pH 9 and short reaction time recovering a biological activity of about 80%. AFM microscopy was used to study and confirm the Abs oriented immobilization on lipase-coated magnetic particles and the final achieving of a highly active and recyclable chimeric immune-sensor. This direct technique was demonstrated to be a powerful alternative to the indirect immunoactivity assay methods for the study of biomacromolecule oriented immobilizations.

Keywords

Magnetic particles, Antibodies oriented immobilization, Lipase, Atomic-force microscopy, protein modifications, Biosensors.

Introduction

The interest in antibodies immobilization on magnetic particles is increasing in biotechnological and biomedical applications as cell sorting, bio-separation, protein purification, immune-sensors, *in vivo* diseases detection and treatment etc.[1-3.] Antibody (Ab) is a glycoprotein composed by hundreds of amino acids arranged in a tridimensional order forming the known Y-shaped basic structure (Figure 1a).

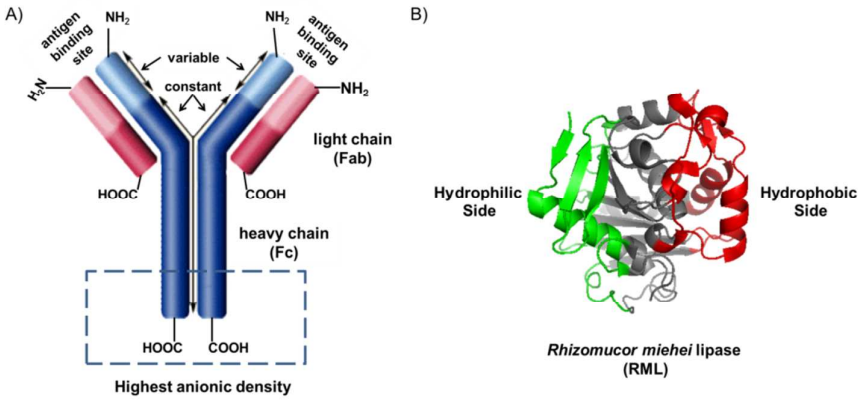


Figure 1. Representation of a) generic antibody structure; b) Lipase from *Rhizomucor Miehei* (PDB file:4TGL).

It is composed by two main parts: the heavy chain (Fc), commonly repeated for the same type of Ab, and two light chains (Fab) responsible of specific antigen recognition that is different for each Ab family.[4-5] Many approaches to immobilize Ab on solid supports have been described. Most of them are based on a random immobilization of these glycoproteins on particles with high yield but low recovered biological activity due to steric hindrance of their active sites (on Fab chains) or inactive lying orientation.[6] To solve this problem, a site-directed Ab immobilization *via* Fc fragment is advisable.[7] In fact, especially for the nanobiomedicine in general, this aspect is becoming each day a more crucial parameter for the development of novel powerful and sensible biosensors.[7b,7c] In this direction, three main general strategies for the oriented Ab immobilization have been developed in the last years: (i) *via* Fc region binding proteins as A and G proteins, (ii) *via* chemical or enzymatic oxidation of oligosaccharide moieties placed on Fc region to aldehyde and subsequent

coupling with amine or hydrazine terminated supports, (iii) *via* Ab fragments after reduction of disulfide groups there placed and subsequent immobilization with sulfhydryl groups generated on a heterogeneous supports.[7,8] These methods are more orienting and specific but characterized by non-covalent attachment in some cases, inactivation of Fab fragment due to undesired modification of some crucial functional groups, increase of the dimensions of the immobilized layer and increase of potential cross-reactivity. [8-10]

Hence, in order to design an efficient immune-sensor, two key parameters should be considered: Ab functional orientation and final system surface inertia. Especially the correct immobilization of the Ab is highly important to maintain its biological activity in recognizing high size antigens such as cells or proteins. Therefore a careful choice of the immobilization method is required. To this scope, the recognition part should be immobilized as far as possible from the support surface to avoid steric hindrance problems that can detract the biological activity. Moreover, in certain cases, the correct immobilization of Ab should also permit the cost reduction in immune-sensor preparation (especially when rare Abs are used) thanks to the reduced used amount of Ab expressing the maximum recovered biological activity. Finally, the creation of final completely inert surfaces avoiding nonspecific interactions with target antigen or other biomolecules is another mandatory requirement to be kept in mind in order to avoid false positive responses. The use of magnetic particles as support for the Ab immobilization is highly advantageous because they offer, besides a high superficial area and thus a high load capacity, the possibility to recover the biosensor after its use by a magnet. Nevertheless, many commercial particles possess a hydrophobic behavior and they need the presence of surfactant to form a stable colloidal suspension in aqueous medium. Recently, an innovative preliminary strategy consisting in the reversible coating of hydrophobic magnetic particles with modified lipases as *Janus* molecules was presented.[11] This system was able not only to avoid particles aggregation after surfactant elimination but also to immobilize a protein mixture contained in an *E. coli* cellular extract.

Hence, in the present work, the evolution and optimization of lipase-coated magnetic particle biosupports as key-element for the corrected standing orientation and subsequent covalent immobilization of different Abs without the need of their previous chemical modification is described. The rational of this strategy relies on the oriented immobilization of biologically active antibody by a two-step mechanism consisting in i) a first ionic adsorption between the Ab area that shows the highest anionic density and located in the bottom part of Fc region (due to an high concentration of Asp and Glu residues, together with the presence of terminal carboxy groups of Ab F_c chains) (Figure 1a) and the amino-epoxidated surface of lipases (previously modified by solid-phase based chemical techniques) followed by ii) a covalent bond formation. Additionally, due to the reversibility of the physical adsorption of lipases on hydrophobic particle surface, this strategy allows the recovering and recyclability of the magnetic particles at the end of the life-cycle of the sensor by means of surfactant-mediated desorption of the chimeric lipase-Ab construct.

Immunoglobulin G from human serum (HIgG) and Horseradish Peroxidase antibody (aHRP) were used as models for the oriented immobilization and recovered biological activity studies considering that the Fc part is generally conserved in other antibodies. Furthermore, considering the ability of Atomic Force Microscopy (AFM) to investigate and retrieve the structure, function and topography of biological molecules and their derivative,[12] we successfully applied this technique for the direct analysis of the immune-sensor surface topography and to positively cross-check the good but indirect results of recovered activity through the direct observation of the correct standing orientation of Ab driven by the two-step immobilization protocol here developed.

Experimental

Materials

Immunoglobulin G from human serum, Anti-Peroxidase antibody produced in rabbit, Ethylenediamine, Epichlorohydrin, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *p*-

Nitrophenyl butyrate (*p*-NPB), Aspartic acid, Sucrose monolaurate, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased by Sigma (St. Louis, MO, USA). Coomassie (Bradford) and Bicinchoninic acid (BCA) protein assay kits were purchased from Pierce (USA). 2,4,6-Trinitrobenzensulfonic acid (TNBS) was from Fluka. Octyl-sepharose CL-4B (OS) was from GE Healthcare (Uppsala, Sweden). *Rhizomucor miehei* lipase (RML) (commercial name: Palatase 20000 L) was from Novozymes (Denmark). BCA Protein assay reagent kit was purchased from Thermo Scientific. Polystyrene coated magnetic microparticles SPHERO^{TM} (2.5μm) (MP) were from Sperotech Inc. (Illinois, USA).

Methods

Lipase activity assay. The activity of soluble and immobilized lipases was carried out spectrophotometrically by measuring the increase in absorbance at 348 nm produced by the release of *p*-nitrophenol (*p*NP) in the hydrolysis of 0.4 mM of *p*-nitrophenyl butyrate in sodium phosphate (25 mM) 25°C (ε = 5150 $M^{-1}cm^{-1}$). To initiate the reaction 0.05-0.2 mL of either lipase solution or suspension was added to 2.5 mL of substrate solution. Enzymatic activity (A) is presented as μmol of *p*NP produced per minute per mg of enzyme (IU) under the conditions above described.

Soluble lipase concentration was measured according to Bradford assay[13] using bovine serum albumin (BSA) as reference for soluble lipases whereas to determine immobilized concentration the BCA assay was used.[14]

Immobilization of *Rhizomucor miehei* lipase on octyl-sepharose. To adsorb RML, 1 g of commercial hydrophobic matrix octyl-sepharose (OS) was added to 10 mL of NaH_2PO_4 (10 mM) buffer solution containing different amounts of commercial *Rhizomucor miehei* lipase (RML) (lipase concentration in crude extract: about 1.5 mg_{lipase}/mL, measured by Bradford assay). The final suspension was kept on mechanical stirring at room temperature during different times, as outlined

in the main text. To check the immobilization course, periodically, the activity of the suspension and supernatant was analyzed by using *p*NPB assay as above described. The immobilization yield was calculated by means of the equation:

$$\text{Immobilization yield (\%)} = [1- (A_{supernatant})/(A_{reference})] \times 100$$

with A= activity value in IU retrieved by lipase activity assay.

Chemical amination of immobilized lipases. Briefly, 1 g of octyl-sepharose containing immobilized RML was added to 10 mL of 1 M ethylenediamine aqueous solution with pH 4.75, under mechanical stirring. Solid 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added to the suspension to a final concentration of 10 mM and the final pH set to 4.75. After 90 min of incubation at 25 °C, the enzyme preparation was filtered and firstly washed with 25 mM NaH_2PO_4 pH 7.5 buffer and then with abundant distilled water.

Pycrilsulfonic acid or TNBS assay. To evaluate the presence of the primary amino-groups TNBS method was used.[15] In particular 0.1 g of support were weighted and 0.4 ml of $NaHCO_3$ (100 mM) buffer solution at pH 10 were added. Then, 0.1 ml of picrylsulphonic acid 5% aqueous solution were added to the suspension and stirred during 10 min. After this time, the support was recovered by filtration and washed 5 times with distilled water and 1 time with $NaHCO_3$ (100 mM) buffer solution at pH 10. The presence of amino groups is confirmed by the red coloration of the support due to the reaction of the pycrilsulphonic acid with primary amino groups of the lipase immobilized on the octyl-sepharose belonging to lysine residues and to the newly generated amino groups.

Epoxidation of amine-modified immobilized lipases. To a solution composed by 16 ml of $NaHCO_3$ (100 mM) buffer pH 9, 4.15 ml of acetone and 4 ml of epichlorohydrin, 1g of aminated RML adsorbed on octyl-Sepharose supports was added at room temperature under mechanical

stirring. The final pH value was set to 9 and the final suspension was kept on stirring at room temperature for 15 h. Subsequently, the immobilized amino-epoxydated lipase was firstly washed with acetone solution (20% v/v) in water (2 x 50 mL) and subsequently with abundant distilled water.

To confirm the succesfully amino modification with epoxy groups the TNBS assay was carried out. In this case, the aim of the assay is to check the disappearance of the red color of the amino epoxydated lipase compared to the sole aminated ones.

Desorption of amino-epoxidated lipases from octyl-sepharose. To 9 mL of NaH_2PO_4 (25 mM) buffer solution at pH 7 containing different amounts of sucrose monolaurate and sodium chloride (when required), 1g of amino-epoxidated lipases (M-RML) immobilized on octyl-sepharose were added. To check the desorption course, the activity of the suspension and supernatant was analyzed by using the lipase activity assay as above described. After that, the desorbed modified enzyme was recovered by filtration.

To remove the surfactant, firstly, NaH_2PO_4 (1M) pH 7 buffer solution was added to the supernatant to obtain a final 100 mM buffer concentration. Subsequently, with the aim to hydrolize the detergent, glyoxyl-aminated *Thermomices lanuginosus* lipase derivative,[16] in a 1:5 w/v proportion, was added to this solution. The final suspension was kept on mechanical stirring at room temperature for 6 h. Once checked the end of the detergent hydrolysis (by HPLC analysis[13]), the supernatant was recovered by filtration and dyalised against distilled water for 15h. The protein concentration of the final purified solutions was measured by Bradford assay.

Unreacted epoxy groups blocking. To block their unreacted epoxy groups, 1 g of M-RMLs (on OS or MPs and with or without Abs bound on their surface) were resuspended in 20 mL of aspartic

acid (3 M) pH 8.5 solution and kept on mechanical stirring at room temperature for 15 h. Afterwards the chimeric derivative was washed 5 times with distilled water.

Immobilization studies of Immunoglobulin G from human serum or Anti-Peroxidase antibody produced in rabbit on amino-epoxydated RML immobilized on octyl-sepharose.

To 10 mL of $NaHCO_3$ (10 mM) solution buffered at different pH values and containing different amounts of both antibodies (antibody amount in crude powder: about 20% w/w in both cases measured by Bradford assay) 1 g of M-RML-OS were added, the final pH set to the desired value and the final suspension was kept on mechanical stirring at room temperature for different reaction times (as described in main text). To check the immobilization course, periodically, the antibody amount of the supernatant was analyzed by using Bradford's assay. After the desired times, to remove all the molecules not covalently bound to the lipase moiety, the antibody-lipase chimers immobilized on OS were recovered by filtration and washed with a 0.5M NaCl solution (3 x 10 mL). The chimeric construct was finally recovered by filtration and washed with distilled water (5 x 10 mL). After that, in the case of aHRP(M-RML-OS) chimeric immune-sensor, before to check its recovered activity by means of the peroxidase activity assay described below, the unreacted amino-epoxy groups were blocked as previously described.

Peroxidase activity assay. HRP activity was determined using H_2O_2 as the oxidizing substrate and ABTS as the reducing substrate. Activity was followed spectrophotometrically by recording the increase in absorbance at 430 nm promoted by the ABTS oxidation product ($ABTS^+$) (ε_{ABTS} = 36,000 M^{-1} cm^{-1}). Experimental conditions: in a quartz cuvette, 2.5 ml of ABTS (1 mM) in NaH_2PO_4 (50 mM) and 20 µl of H_2O_2 (100 mM) pH 6 solutions were mixed at 25 °C. After that, a desired amounst of HRP containing sample were added to start the assay. Enzymatic activity is

presented as μmol of $ABTS^{+}$ produced per minute per mg of enzyme (IU) under the conditions above described.

Theoretical calculation of maximal lipase amount immobilized on magnetic particles. The 6 mg/g theoretical maximal load of lipase on microparticle surface was calculated multiplying the molecular weight of RML (about 30 KDa) and the number of RML molecules needed to obtain a lipase monolayer on magnetic particles surface. This molar ratio (MR) between RML and magnetic particles (MPs) was calculated by means of the following equation:

$$MR = N_{RML}/N_{MP}$$

N_{RML} and N_{MP} are respectively the amount of RML molecules and MPs obtained knowing two critical parameters, particle diameter and RML footprint that have been obtained using the following equations:

$$N_{RML} = A_{MP}/FP_{RML}$$

And

$$N_{MP} = d_{MP} \times V_{MP} \times N_A / MW_{MP}$$

Where:

A_{MP} is the superficial area of the 2.5 μm diameter spherical magnetite particles: $4\pi r^2$, measured in nm^2.

FP_{RML} is the RML footprint and it has been calculated as 4 nm^2. This value was obtained by measurement of diameter of RML open structures (about 2.3 nm) using PyMOL software (PDB file: 4TGL) and considering the RML footprint as the circle area: $A= \pi r^2$.

d_{MP} is the density of the magnetite: 5.15 g/ml.

V_{MP} is the volume of the magnetic particles: $4/3\pi r^3$ measured in ml (considering that 1 $ml=1x10^{21}nm^3$).

N_A is Avogadro number: $6.022x10^{23}$.

MW is the molecular weight of magnetite: 231.54 g/mol.

Preparation of aHRP(M-RML@MP) chimeric biosensor on magnetic particle under optimal conditions. Under gentle mechanical stirring (orbital shaker) and room temperature, 0.100 g of 2.5 μm diameter polystyrene coated microparticles (obtained after removing by centrifugation the commercial supernatant and washing the obtained solid with distilled water) were resuspended in 5 mL of NaH_2PO_4 (5 mM) pH 7 buffer solution containing a 1 mg total amount of pure amino-epoxidated lipase (M-RML). To check the immobilization course, periodically, the lipase amount of the suspension and supernatant was analyzed by using Bradford's assay. After 15 h, about 65% of immobilization was achieved with a final M-RML load of 6.5 mg/g_{MP}. Subsequently, the microparticles coated with the modified enzyme (M-RML@MP) were recovered by centrifugation and washed several times with abundant water. The final modified lipase amount adsorbed on polystyrene magnetic particle surface was cross-checked by BCA assay.

In order to immobilize the aHRP antibody, 100 mg of M-RML@MP were resuspended in 1 ml of $NaHCO_3$ (10mM) buffer at pH 9, containing 0.1 nmol of aHRP and kept on mechanical stirring at room temperature. After 2h, the aHRP immobilization yield was about 90% (by Bradford analysis of supernatant). The chimeric derivative was then recovered by centrifugation (10K rpm, 4ºC and 20 min), washed 3 times with 1 ml of of NaCl (0.5M) solution (no antibody was detected in the recollected supernatants by Bradford assay) and finally resuspended in 2 ml of aspartic acid (3M) solution at pH 8.5 to block the unreacted epoxy linkers. After 15h, the magnetic particles were recovered by centrifugation and washed abundantly with distilled water.

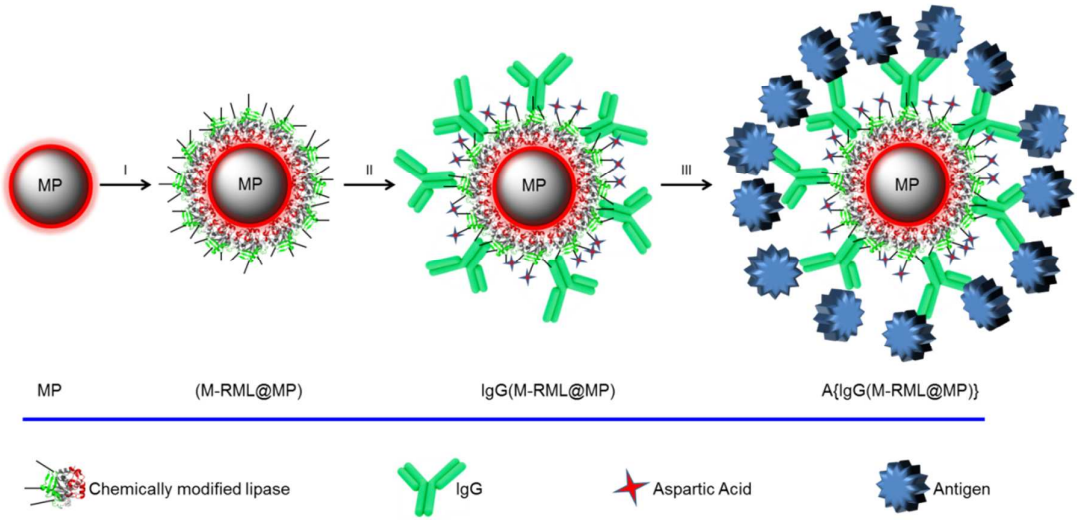
Atomic Force Microscopy Characterization. A model 5500 microscope from Agilent Technologies (Santa Clara, CA) was used for AFM imaging. Measurements were made under ambient conditions in the dynamic mode, using Olympus rectangular silicon nitride cantilevers

(RC800PSA, 100 × 20 μm) with a spring constant of 0.38 N m^{-1}, an estimated tip radius of 20 nm, and a resonant frequency close to 70 kHz. The scanning rate was ca. 1.5 Hz. When necessary, the images were second order flattened to correct for image "bow" due to nonlinearity in the piezoelectric scanner. The surface root-mean-square roughness, Rq, was evaluated with the software Picoimage, from Agilent Technologies, in at least five different 2 x 2 μm^2 scans for each sample following removal of artifacts and averaging. The microsphere dispersions in DI water were directly drop casted in the mica surfaces and let completely dry.

Results and Discussion

Optimized preparation of modified lipase-coated magnetic particles.

As previously commented, the starting approach to produce and characterize the chimeric biosensor was initially based on the general idea described in Scheme 1.[11]



Scheme 1. General strategy for the preparation of oriented Abs/lipase chimeric immune-sensor on magnetic particles (MP). I) Immobilization on hydrophobic magnetic particles of lipases previously modified on solid phase; II) oriented two-step immobilization of antibodies and surface inhertization; III) Antigen/chimeric immune-sensor final complex.

The main rational underscored by this strategy is based on the ability of lipases to be adsorbed on hydrophobic supports through their characteristic hydrophobic zone (known as lid) allowing by this way the solid-phase chemical modification of their opposite hydrophilic side (Figure 1b). Hence, the handful high-scale production of pure modified lipases clearly results as the critical bottle-neck to permit their wide application as advanced multifunctional biosupport. Consequently, the maximum enzyme loading of a commercial lipase from *Rhizomucor miehei* (RML) that could be adsorbed on the commercial octyl-Sepharose hydrophobic support (OS) was firstly studied (Scheme S1). Increasing amounts of crude lipase were then offered to 1 g of support (Table 1).

Table 1: Immobilization studies of RML on octyl-sepharose.

entry	Offered RML (mg/g)	Immobilized RML (mg/g)	Immobilization yield (%)
1	1.5	1.5	100
2	10	9	90
3	50	44	88
4	100	71	71
5	125	69	55.2

The best achieved result was the immobilization of about 70 mg of RML per gram of support after 48h at room temperature (checked by enzymatic activity and Bradford´s method) (Table 1, entry 4). This long immobilization reaction time (48h) was arbitrarily selected to ensure the maximum lipase adsorption.

After its adsorption on the OS hydrophobic support, the lipase was subsequently chemically modified in order to generate on its surface the orienting heterofunctional linkers for the antibody immobilization. The activation consisted in the chemical epoxidation of the amine groups naturally present on the lipase surface. Moreover, in order to maximize the outcome of covalent immobilization, the obtainment of a chimeric lipase activated on its surface by the highest density

of epoxy-groups was a main objective. Hence, considering the poorness of native amine side chain groups (lysines: 7) compared with the carboxylic ones (aspartic and glutamic acids, 15 and 13 respectively) on RML surface, chemical amination of these lasts was performed *via* amide formation using ethylenediamine and carbodiimide as coupling agent (Scheme S1). The quantitative chemical modification was successfully achieved using 10 mM carbodiimide (the reaction outcome was checked with the TNBS colorimetric assay). Higher concentrations of coupling agent did not report better results. After their creation, the amino groups were then treated with epichlorohydrin to finally create the key amino-epoxy groups able to orient and covalently react with different Abs (Scheme S1).

For the subsequent immobilization on hydrophobic magnetic particles, the modified lipase (M-RML) molecules must be desorbed from the porous support (Scheme S1). To this scope, lauryl sucrose (L.S.) was chosen as surfactant for its advantage to be hydrolyzed in the presence of a lipase catalyst allowing its easier elimination from the reaction medium, especially if present in high concentration.[16]

Considering that the high density of amino-epoxidated lipase molecules reversibly adsorbed on the support may promote not only hydrophobic lipase-support interactions but also other undesired interactions (mainly ionic) such as lipase-lipase or lipase-support, the optimization of the crucial parameters for lipase desorption (i.e. concentration of lipase in the support, surfactant concentration, sodium chloride concentration or different dilution proportions) were then studied. As reported in Table 2, the lipase concentration highly affected the desorption yield (entry 1 and 2). This result clearly indicates the presence of lipase-lipase interactions promoting the reinforcement of the total adsorption, although at low lipase concentrations, the desorption yield was quite high.

In order to improve the yield of this step using high lipase amounts, other parameters were then considered and optimized. Diluting the suspension by a 2 fold factor, a desorption yield of 34% (Table 2, entry 3) was promoted. Maintaining this parameter and increasing the lauryl sucrose

concentration until 3%, a further improvement up to 60% desorption yield was obtained (Table 2, entry 5).

Table 2: Desorption studies of modified RML from octyl-sepharose.

entry	$mg_{RML}/g_{sepharose}$	L.S. (% w/v)	NaCl (M)	Vol. (ml)	Desorption yield (%)
1	1	1	-	10	62
2	70	1	-	10	18
3	70	1	-	20	34
4	70	2	-	20	48
5	70	3	-	20	60
6	70	4	-	20	59
7	70	1	0.5	20	49
8	70	2	0.5	20	73
9	70	3	0.5	20	72
10	70	2	1	20	69

Higher concentrations did not enhance these results (Table 2, entry 6). On the other hand, the addition of 0.5 M of NaCl increased the M-RML desorption until 73% of the initially adsorbed amount (Table 2, entry 8). This result confirmed the existence of ionic lipase-lipase interactions. Higher salt concentrations were also used without significant improvements (Table 2, entry 10). After desorption, the surfactant present in the lipase suspension was removed applying an enzymatic protocol (a first surfactant hydrolysis catalyzed by an immobilized *Thermomices lanuginosus* lipase followed by dialysis) previously developed.[16]

Finally, the desorbed and purified activated lipases were immobilized on polystyrene magnetic particles (MP) by means of hydrophobic physical adsorption (Scheme 1). Considering the importance of the complete masking of the hydrophobic surface of the particles to obtain a final inert system, the complete recovering of their surface with lipase molecules was studied. The best results were obtained re-suspending 100 mg of commercial magnetic microparticles in a solution containing 1 mg of purified M-RML and incubated at room temperature. After 15 hours, the immobilization yield of M-RML on MP was about 65% corresponding to 6.5 mg of lipase for gram of magnetic support (checked by enzyme activity and Bradford). The same immobilization yield was obtained reducing the incubation time to 4 h (data not shown). These results generally agreed with the theoretical maximal load of 6 mg_{RML}/g_{MPs} calculated considering the average diameter of the particles (about 2.5 μm corresponding to a superficial area of 19.6 $x10^{-6}$ nm^2) and the footprint of RML (quoted as 4 nm^2) (see Experimental section for more details).

Oriented Ab immobilization on modified lipase surface.

As previously commented in the Introduction, the reactive amino-epoxy groups are expected to be adequate for antibody immobilization considering that the Fc region is the richest in negative charged amino acids.[5,7] The designed immobilization strategy was based on a mechanism involving two precise steps: i) a first *intermolecular* ionic adsorption of negatively charged Fc region of Ab driven by the positive charges of amino-epoxy groups artificially created on the surface of modified lipases; ii) *intramolecular* covalent immobilization of the previously adsorbed biomacromolecules through covalent reaction of epoxy groups with nucleophiles groups of Ab present in Fc region (Scheme S2).[11] Consequently, important parameters such as pH, incubation time, ionic strength or offered antibody amount were deeply investigated and optimized.

In order to validate the general applicability of the methodology for a wide range of Abs, HIgG and aHRP were used during the different optimization steps expected by the proposed

strategy. Hence, HIgG was firstly used to identify the optimum pH value to successfully enhance the immobilization reaction. To this scope the M-RML-OS derivative was incubated during 72h with a 10 mg/ml of HIgG solution buffered to different pH values with low concentration of $NaHCO_3$ to promote the first step of ionic adsorption (Figure 2).

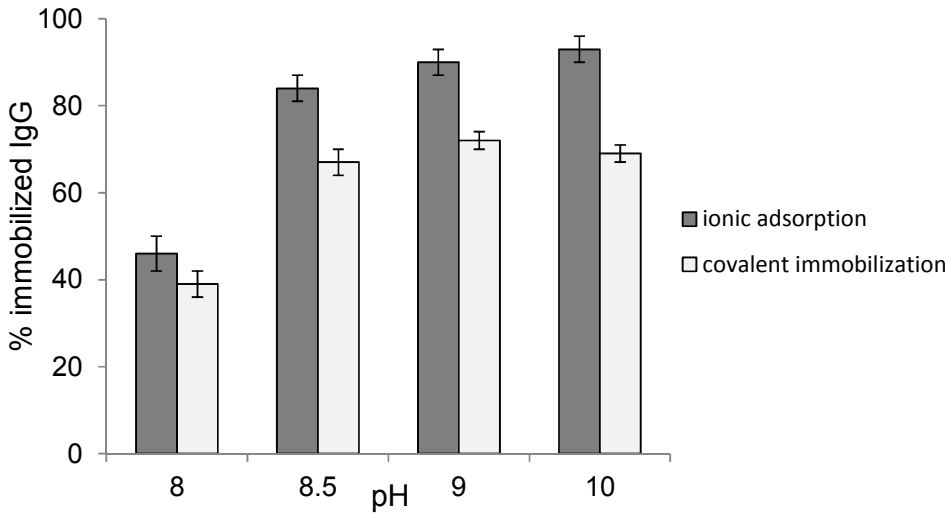


Figure 2. pH-dependent two-step immobilization of HIgG on M-RML-OS (mean values ± se; n = 6).

Being the entire immobilization process (adsorption *plus* covalent immobilization) finely tuned by pH variations, we found that the HIgG adsorption directly increased with a pH increase. This evidence is coherent with the high isoelectric point of the antibodies (higher than pH 7), confirming the need of a basic reaction medium to create a net negative charge on the HIgG surface responsible of its first ionic adsorption on amino-epoxy groups. Considering that the pKa of the amine groups chemically generated on the lipase surface was around 10, with pHs higher than this value these groups will be uncharged and consequently unable to interact with IgG negatively charged molecules. Among all the results obtained testing different pH values, the highest HIgG absorption (checked by Bradford assay of supernatant) was achieved when the pH was set around to a value of 9 or 10 (Figure 2). After the first step optimization, the subsequent covalent immobilization was

then studied. As in the case of the ionic adsorption, the covalent immobilization was improved by higher pH values because at these conditions the nucleophile groups on HIgG surface became more reactive toward the epoxy groups. The maximal reactivity was checked by washing the final derivative, incubated at different times and pH values, with a high ionic strength solution (0.5 M NaCl) until no significant amounts of IgG were leaked to the supernatant. By this way, the formation of a stable covalent linkage between Ab and M-RML surface was confirmed. Thanks to these studies the optimal pH value to promote the covalent oriented immobilization of Abs on lipase surface after the first orienting ionic adsorption was found to be 9 (Figure 2). Higher pHs did not increase covalent immobilization probably because of the low stability of the epoxy groups at strong basic pH values.

Once optimized the pH conditions for HIGg covalent immobilization on lipase modified surface, in order to indirectly check the Ab orientation by means of the analysis of its recovered activity, an antibody showing biological activity as aHRP was used. To test its activity, the enzyme horseradish peroxidase (HRP) was used as antigen and the 100% of the biological activity was theoretically considered as the binding of two peroxidase molecules per aHRP molecule. To this scope, as previously reported in the Introduction, in order to avoid any possible nonspecific interactions with target antigen or other biomolecules, the inertia of the final system surface was also studied. To this scope the epoxy-groups of the modified lipase (without the Ab) were blocked with aspartic acid in order i) to hamper the possibility to still follow the immobilization reaction with the immobilized Abs once reached the optimum results and ii) to eliminate nonspecific adsorptions of antigen on the final system surface due to the excess of artificially inserted positive charges (Scheme 1). Hence, after incubation of M-RML-OS construct in 3M Aspartic acid solution at pH 8.5, the optimal ionic strength preventing the unspecific adsorption of peroxidase to Asp-M-RML-OS system, after their co-incubation, was investigated. The best result was found to be 250 mM NaH_2PO_4 buffer pH 7 (Figure S1). Hence, the use of the aspartic-blocked derivative in these

conditions allowed guaranteeing that the capture of the antigen will be promoted only by the Ab and not by unspecific physical adsorption on the support.

Once established the best conditions to check the recovered biological activity, the immobilization studies using Anti-Peroxidase antibody produced in rabbit were then performed. Firstly, the impact of the incubation time of aHRP on recovered activity was analyzed. Thus, about 20 nmol of aHRP were offered to 1 gram of octyl-sepharose coated with the modified lipases. As reported in Table 3, the increase of immobilization time (from 4 to 72 h) improved the immobilization yields (entries 1-3) but also reduced the recovered biological activity (up to 1%, checked with the peroxidase activity assay of supernatant containing the peroxidase solution after its incubation with blocked chimeric immune-sensor on solid support), with only 0.2 $nmol_{HRP}$/g bound (Table 3, entry 3).

Table 3. Immobilization studies of aHRP on M-RML-OS.

Entry	Offered aHRP (nmol)	Time (h)	Immobilized aHRP (nmol)	Immobilization Yield (%)	Recognized HRP (nmol)	Recovered Activity (%)
1	20.5	4	4.1	20	0.82	10
2	20.5	24	6	29.2	0.33	2.75
3	20.5	72	9.23	45	0.2	1
4	4.1	4	1.93	47	1	26.3
5	2	4	1.81	90.5	1	27
6	1	4	1	>99	1.77	89
7	0.5	4	0.5	>99	0.87	85.3
8	1	2	0.99	99	1.73	87
9	1	1	0.69	69	1.16	85
10[a]	1	24	1	>99	0.04	2

[a] Incubation at pH 10.5.

This result can be explained by two possible effects: i) longer incubation times promoted higher immobilization yield of aHRP thus increasing the Ab density and consequently generating steric hindrances on lipase surface finally hampering the accessibility of the peroxidase antigen (a quite

high volume enzyme, Figure S2) to Fab region; ii) due to longer incubation times, more covalent bonds with the support surface were formed, bearing to a progressive less active lying orientation of Ab with a minor normal angle to the lipase supporting surface. To shed light on our hypothesis, these effects were studied separately. Thus, in a first set of experiments, different amounts of aHRP were offered to the support and the immobilization time was fixed to 4 h. The results indicate that decreasing the offered antibody amount, the biological activity was increased (Table 3, entries 1 and 4-7) confirming that when the density of the immobilized aHRP was too much high, undesired steric hindrances were generated. In term of aHRP immobilization amount, the maximum value was found to be about 1.9 nmol of antibody per g of derivative (entry 4). Nevertheless, in terms of recovered activity, the best result (about 1.8 nmol of HRP x g^{-1} derivative, almost 90%) was achieved when 1 nmol of aHRP was offered to 1 g of amino-epoxidated RML adsorbed on octyl-sepharose (Table 3, entry 6).

Under these optimized conditions (pH 9, 1 nmol of offered aHRP/g_{MP} and RT) the incubation time was reduced at the half (2h) and no significant differences in recovered activity were observed (Table 3, entry 8). Further reductions in incubation times reduced the immobilization amount of aHRP and the recovered activity (Table 3, entry 9). Very notably, when the best Ab(M-RML-OS) derivative loaded with 1 nmol of immobilized Ab for 2 hours (Table 3, entry 6) was incubated for 24 hours at pH 10.5 (in order to promote the formation of more covalent linkages), the recovered activity decreased up to 2% indicating that the increase of interactions between Ab and the chimeric support promotes the reduction of the recovered biological activity of the antibody (Table 3, entry 10). These results confirm the importance to finely control the immobilization protocol to achieve the best standing oriented immobilization of antibodies. In fact, it has been shown that parameters like incubation times (to promote an adequate but not excessive covalent immobilization after the first ionic adsorption) or the density of immobilized antibodies (to overcome the steric hindrance problems) have been revealed to be crucial.

Preparation of chimeric Ab biosensors on magnetic particle.

Once optimized the preparation of an inert and highly active immune-sensor on the model support octyl-sepharose, the preparation of chimeric and inert lipase-based biosensor on the magnetic particle was studied (Scheme 1). As previously commented, the complete masking of the hydrophobicity of the surface of the magnetic particles is mandatory in order to obtain inert systems and stable colloidal suspensions in water. To this scope, magnetic particles coated with the maximal load of chimeric lipase (6.5 $mg_{RML}/g_{particles}$) were prepared (M-RML@MP).

In order to immobilize the aHRP antibody, 100 mg of M-RML@MP were resuspended in $NaHCO_3$ (10mM) pH 9 buffer solution containing 0.1 nmol of aHRP and kept on gentle mechanical stirring at room temperature (Scheme 1). After 2h, the aHRP immobilization yield was about 90% (by Bradford analysis). The chimeric derivative was then recovered by centrifugation, washed 3 times with NaCl (0.5M) solution (no antibody was detected in the recollected supernatants by Bradford assay) and finally resuspended in aspartic acid (3M) solution at pH 8.5 to block the unreacted epoxy linkers. After overnight, the recovered activity was checked by means of peroxidase immunoassay retrieving an almost 80% value.

Direct antibody orientation analysis through Atomic Force Microscopy (AFM).

Generally in literature, as well as also in this work, the most used and valuable strategy to check the orientation of antibodies after their immobilization is the evaluation of the recovered biological activity by means of immunoassay. Despite its recognized validity, this assay still follow being an indirect way to check the Ab orientation and no direct evidences of their distribution and orientation could be obtained. Considering the special ability of atomic force microscopy (AFM) to determine and visualize the topography of a wide range of surfaces at nanometric scale, this technique was used to directly analyze the surface of lipase-antibody coated MPs after each step of their

preparation protocol (Figure 3) with the final aim to validate it as powerful on-line tool to analyze the orientation/activity relationship during biomacromolecule immobilization.

Differently than other similar research papers,[17,18] the generated chimera was analyzed directly on the coated microspheres without recreating the chimeric construct on the modified planar glass slides that normally are used as sample support for AFM analysis. This decision was kept considering the geometrical differences existing among the supports (spherical microsphere *vs* planar slide) and, mainly, to avoid the possibility to alter the Ab orientation if promoted on a different behavior, creating by this way a final unreal artifact. To the best of our knowledge, this work represents the first example of an Ab orientation topographic study on microbeads surface performed by AFM.

First, with the aim to check their surface roughness, a general analysis of uncoated commercial MPs was performed (Figure 3, column I).

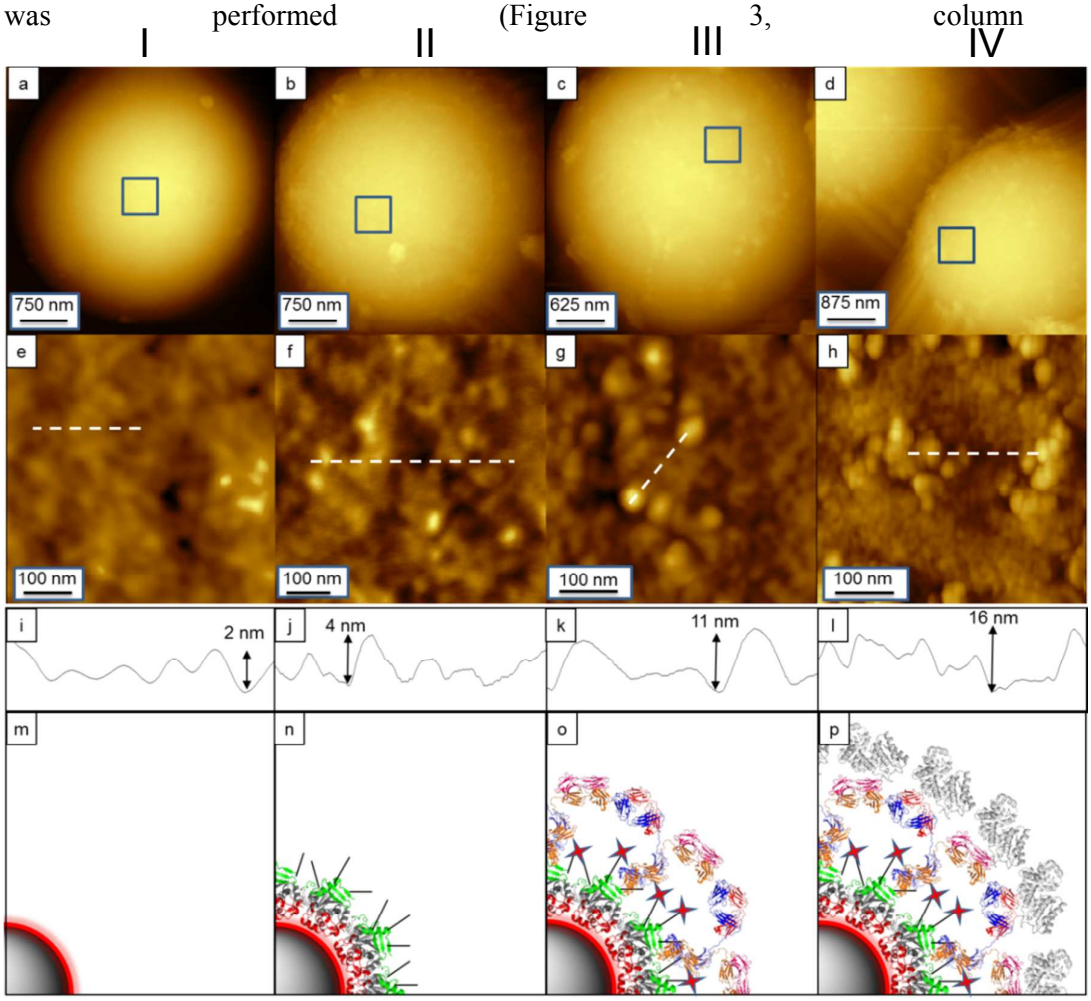


Figure 3. AFM analysis of each step of the proposed strategy. Column I) magnetic hydrophobic particle (a, e, i and m); Column II) M-RML@MP (b, f, j and n); Column III) aHRP(M-RML@MP) (c, g, k and o); Column IV) HRP[aHRP(M-RML@MP)] (d, h, l and p).

As observed in figures 3a, 3b, 3c and 3d, the microsphere's surface is populated by some huge structures with heights ranging from 50 nm to more than 200 nm that are attributed to surface heterogeneities, broken parts of the microspheres coat (as demonstrated by their presence also in commercial MP (column I) and/or to molecular aggregates. However, the rest of the surface of the microspheres (more than 90 % of the total surface) was found to be rather homogenous and flat

enough for the AFM characterization of the molecular architectures. The magnification of the MPs surface exhibited a peak-to-valley height smaller than 2 nm (Figure 3 e, i and m) and a surface R_q (extracted from 5 different areas of the MP of 200x200 nm^2) of about 0.47 nm. Subsequently, the AFM analysis of microspheres coated by modified lipases was performed (Figure 3, column II). The results showed a rougher surface with an Rq of 0.91 nm and peak-to-valley heights of about 4 nm, both compatibles with the presence of a lipase monolayer. In fact, the 4 nm height value was obtained by measurement of the dimensions of RML open structure using PyMOL software (PDB file: 4TGL) and it results in agreement with other AFM-mediated characterization of similar lipases (Figure 3 f and j).[17] To study the oriented immobilization of antibodies, two different samples of Ab(M-RML@MP) were prepared and analyzed: the sample characterized by the best recovered immune-activity (80%) obtained after 2h incubation (S-1) and the same sample but incubated for longer times (24h) and higher pH values (10.5) and showing a worse recovered immune-activity (about 3%, S-2). The surface of the MPs obtained by both methods presented a series of globules of different sizes (Figure 3, column III for S-1). After a distribution analysis of the height of 15 different globules for each sample, a Gaussian distribution centered in 9 nm for S-1 and 5 nm for S-2 was found in each case (Figure 4a and 4b).

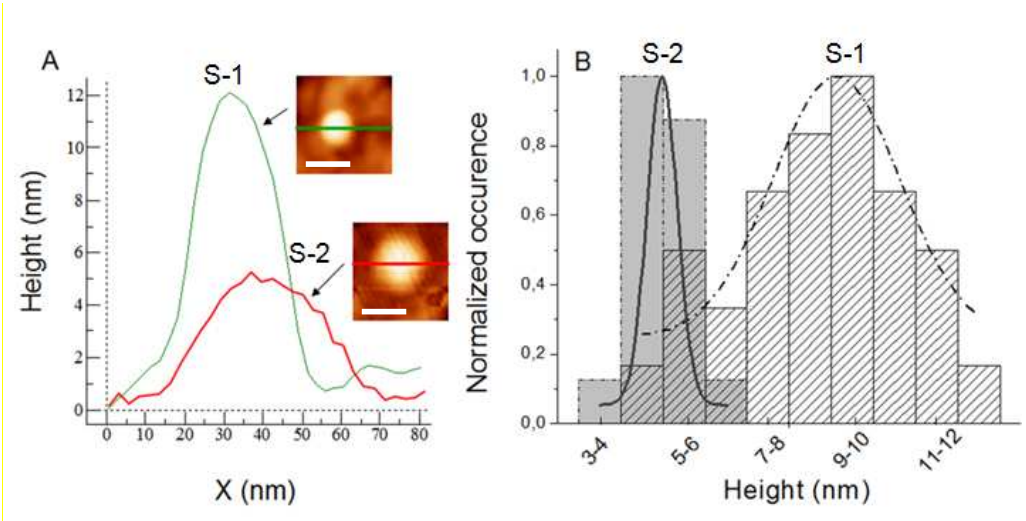


Figure 4. Distribution of HRP orientation after immobilization on M-RML@MP surface. a) Topography (the scale bars correspond to 40 nm). b) Statistic normalized height distribution.

Considering the IgG dimensions (Figure S2a, PDB file: 1IGT),[18] these results clearly indicate that the antibodies immobilized by our optimized protocol here proposed and presenting the highest recovered activity (S-1), in their vast majority possess a standing orientation with a higher normal angle to the surface (almost perpendicular) if compared to S-2. In fact, in this last case, the IgGs have been oriented and immobilized with their longest axe almost parallel to the lipase surface explaining, by this way, their lowest height and the low recovered immune-activity. This evidence is concordant with the experimental protocol applied considering the stressing incubation conditions (pH 10.5 and 24h) of IgGs with M-RML@MPs in the case of preparation of S-2. In fact, as previously described, the IgGs can form many covalent linkages with the epoxy groups present on the lipase surface and finally taking their lying orientation. It is noteworthy that the Gaussian curve for S-2 is much sharper than that obtained for S-1, indicating that almost all the IgGs molecules possess this incorrect orientation (Figure 4b). Finally, the AFM analysis of S-1 biosensor after immune-activity assay clearly indicates as the surface gave rise to larger globules with heights comprised between 15 and 20 nm (Figure 3, column IV), corresponding to the addition of peroxidase molecules on the immobilized antibodies surface, accordingly to HRP dimensions (Figure S2b, PDB file: 1GWU). All these results, obtained by a direct analysis of antibodies orientation, are fully concordant with the indirect evidences retrieved through the recovered immune-activity assay confirming the effectiveness of AFM as general and alternative precise technique to cross-check the immobilization orientation of antibodies or other biomacromolecules on modified magnetic particles.

Conclusions

In sum, a novel optimized and handful strategy for the oriented immobilization of antibodies on magnetic particles permitting to achieve highly active chimeric biosensors was described. It is based on specific and covalent attachments that permit to conjugate Abs in their correct standing orientation through a modified lipase-based support. This strategy permits to recover the maximum biological activity of antibody offering suitable amounts of them and without their previous chemical modification. The use of solid-phase strategies based on the use of porous support with a high internal surface (octyl-sepharose) allowed the large scale production of the chemically modified lipase as the key supporting and orienting element (up to one order of magnitude per gram of support against the use of non-porous nanoparticles). Furthermore, due to this solid-phase strategy, even any possible aggregation of lipase molecules due to the use of bifunctional cross-linkers is prevented and, furthermore, the highly loaded final derivative can be stored at 4°C during long times.

This amino-epoxidated lipase was shown to be the key element for the design of a highly active chimeric immune-sensor that permitted the standing immobilization of different Abs. Various parameters as the Ab offered amount, pH and reaction time resulted crucial to recover the maximum biological activity on magnetic particles (almost 80%). The different Ab orientations generated by the different immobilization protocols were also successfully investigated using a direct technique as AFM demonstrating its usefulness as powerful and precise on-line technique for the study of orientation of biomacromolecules during their immobilization on different solid surfaces.

Associated Content

Supporting Information

Supporting Figures and Schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

Acknowledgements

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TOC

Handful Oriented Immobilization Of Antibodies On Chimeric Magnetic Particles: Direct Correlation of Biomacromolecule Orientation with Biological Activity By AFM Studies.

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