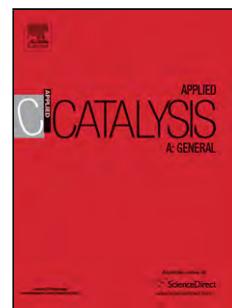


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Magnetic separation and high reusability of chloroperoxidase entrapped in multi polysaccharide micro-supports

Sonia García-Embido,^{a,b,†} Francesca Di Renzo,^{c,†} Laura De Matteis,^{b,d,*} Nicoletta Spreti,^c and Jesús M. de la Fuente^{a,b}

^a Instituto de Ciencia de Materiales de Aragón (ICMA), CSIC - Universidad de Zaragoza, Edificio I+D, calle Mariano Esquillor s/n, 50018 Zaragoza, Spain.

^b CIBER-BBN, Instituto de Salud Carlos III, Madrid, Spain

^c Department of Physical and Chemical Sciences, University of L'Aquila, Via Vetoio, I-67100 Coppito, L'Aquila, Italy.

^d Instituto de Nanociencia de Aragón (INA), Universidad de Zaragoza, C/ Mariano Esquillor s/n, 50018 Zaragoza, Spain.

*Corresponding author: lauradem@unizar.es

† these authors contributed equally

Highlights

- Magnetic micro-supports were developed based on a multi polysaccharide shell.
- All supports were characterized in terms of chemical stability under reaction conditions.
- Chloroperoxidase was successfully entrapped in multi polysaccharide magnetic supports.
- Leakage of the enzyme was observed with single-shell polysaccharide coating.
- High CPO reusability was measured with a polymeric double-shell magnetic micro-support.

Keywords: Chloroperoxidase; polysaccharides; enzyme immobilization; reusability; magnetic nanoparticles; micro-supports.

Abbreviations: PDA = magnetic nanoparticles coated with polydopamine; CS = magnetic micro-supports coated with single chitosan shell; ALG = magnetic micro-supports coated with single alginate shell; CS-ALG = magnetic micro-supports coated with a chitosan-alginate double shell; ALG-CS = magnetic micro-supports coated with an alginate-chitosan double shell; CS-BS3 = magnetic micro-supports coated with a chitosan-alginate double shell and covalently crosslinked with BS3); MMS = magnetic micro-supports.

Abstract

Enzyme immobilization on magnetic supports represents a great advantage for the industrial application of enzymatic catalysis since it allows an easy recovery of the catalyst, avoiding any contamination of the product by residual enzyme. Iron oxide nanoparticles are very useful for this purpose. Using a polymer to diminish the interaction between the magnetic cores themselves, can improve the colloidal stability of the support and prevent any interaction with the environment that would affect both support properties and enzyme stability. For this reason, in this work different magnetic micro-supports, based on polydopamine-coated iron oxide nanoparticles with a multi polysaccharide shell, have been developed. These supports have been used to immobilize chloroperoxidase, a very interesting enzyme, able

to catalyze many reactions of large-scale interest, but whose application is limited by its sensitivity to reaction conditions. The multi polysaccharide shells of the supports were obtained through a combination of chitosan and alginate. An in-depth analysis of physicochemical and catalytic properties of all the developed magnetic supports is reported. CPO was successfully immobilized with an efficiency of entrapment between 92% and 100% in the case of supports with chitosan in the interior or outer shell respectively. A very good chemical stability of the support under reaction conditions was observed in the case of an interior shell of alginate and an outer coating of chitosan, together with an excellent reusability of the immobilized enzyme, that was recycled to catalyze up to 25 consecutive reaction cycles.

1. Introduction

Enzymatic catalysis represents a valuable tool for industrial applications. Nevertheless, in some cases its use on large scale could be a complicated issue due to catalyst sensitivity to reaction conditions and high costs of enzyme production [1]. These limitations can be overcome through enzyme immobilization on solid supports to allow the recovery and reuse of the catalytic system [2–8]. The improved stability of the enzyme to environmental conditions, i.e. presence of organic solvents, temperature and pH, allows a longer lifetime of the catalyst with respect to the non-immobilized enzyme. The easier the handling and recovery of the enzyme, the simpler is the downstream process and the better the economy of the process. Moreover, in the case of using a supported catalyst, the reaction product is not contaminated by any residual of the enzyme, an aspect that gained a great importance in food and pharmaceutical industrial applications.

The choice of a suitable support allows maintaining the catalytic properties of the enzyme after its immobilization and maximizing the efficiency of the conversion process [4].

Enzyme immobilization on nanomaterials represents one of the most forefront research fields in biocatalysis, biosensing and biomedical applications thanks to the unique properties that the small size confers to these supports. An improvement in enzyme stability and catalytic performances can be obtained through enzyme immobilization on nanoparticles, probably rigidifying them and preventing their denaturation in the recycling process. Among all kinds of nanoparticles, magnetic ones offer a great advantage for the applicability of the catalytic system on large scale, since they can be easily separated from the medium at the end of reaction process and recovered for their reuse thanks to their fast response to an applied magnetic field [2–5]. In the last few decades, they have acquired importance as carrier for binding proteins, enzymes, antibodies and drugs, for biomedical and biotechnological applications [2,9–11].

In many cases it has been found that coating and surface modification of these inorganic nanoparticles allow to control the interaction between the magnetic cores themselves and interactions with the environment, improving a better colloidal stability of the support and so a higher activity of the immobilized biomolecule [10,12–14]. Polymeric coatings

are widely used to increase stability and biocompatibility of magnetic nanoparticles and also to preserve their magnetic properties. In fact they can avoid aggregation effects due to magnetic or surface interactions and they can also protect the magnetic core from direct exposure to reaction conditions that could lead to any degradation of the inorganic core. Natural polymers, as polysaccharides, are used as coatings for inorganic nanoparticles since they have the advantage over synthetic ones of being abundant in nature, biodegradable and cheap [12].

Natural polymers were also widely used for the coating of magnetic nanoparticles for enzyme immobilization. Chitosan-coated magnetic nanoparticles were employed to immobilize glucose-6-phosphate dehydrogenase [15] and pectinase [16], while chitosan-collagene or alginate coating were used for lipase from *Candida rugosa* immobilization [17,18]. Among natural polymers, both chitosan and sodium alginate are biodegradable, non-toxic, biocompatible polysaccharides [19–21]. While chitosan is rich in amino groups, which confer positive charges to the polymeric chains at pH values below 6.0, sodium alginate is a negatively charged polymer due to the presence of carboxyl groups. The presence of such functional groups on their structure allows the interaction with biomolecules of interest. Both polysaccharides have been used for the coating of magnetic nanoparticles to obtain biocompatible supports.

Chloroperoxidase from *Caldaromyces fumago* (CPO), a highly glycosylated heme enzyme, possesses a great industrial interest since it is able to catalyze many reactions of large-scale interest, i.e. sulfooxidation, epoxidation and oxidative halogenation. Nevertheless, this enzyme is very sensitive to high concentrations of oxidants, which limit its industrial applications [22].

These drawbacks can generally be overcome by immobilization of the enzyme. Different supports have been employed to improve CPO catalytic efficiency. Silica materials [23–25], iron oxide magnetic nanoparticles [26,27], Au@Fe₃O₄ nanoparticles [28], nanostructured titanium oxide materials [29,30], ZnO nanowire/silica composite [31], alumina nanorods [32], an acrylic-based material (Eupergit® C) [33] and graphene oxide nanosheets [34] are some of the most recently used carrier from the literature.

In our previous papers, hybrid polymer-silica matrices, synthesized by sol-gel method, were found to be excellent supports for CPO immobilization by entrapment [35,36]. In particular, chitosan-silica composites were found to be the most effective supports to improve biocatalyst reusability, up to 18 consecutive reaction cycles [36]. To further investigate the applicability of chitosan-based supports on CPO reusability, in this work, a biodegradable magnetic support for CPO immobilization has been developed through the combination of magnetic iron oxide nanoparticles and a multi-shell support of chitosan and sodium alginate.

A nanoemulsion-based core further coated with polysaccharide multiple shells has been developed to encapsulate magnetic nanoparticles to obtain a magnetic support for CPO entrapment [37]. Magnetic nanoparticles were firstly

coated with polydopamine (PDA), to stabilize them and favor the following interaction with the encapsulating system [18,38]. Different magnetic supports were obtained for CPO immobilization in polymer shells by combining chitosan and sodium alginate. After physicochemical characterization and catalytic tests, the optimal support for catalyst immobilization in terms of easy recovery and high reusability was selected in view of large-scale applications.

2. Experimental

2.1 Materials

Chloroperoxidase (CPO, EC 1.11.1.10) from *Caldariomyces fumago*, as a crude suspension (26,776 U/mL), Monochlorodimedone (MCD, 2-chloro-5,5-dimethyl-1,3-cyclohexanedione), alginic acid sodium salt, Span® 85 (sorbitanetrioleate) (Croda International PLC, Cowick Hall Snaith, UK), oleic acid, chitosan (medium molecular weight), iron(II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) and dopamine hydrochloride were obtained from Sigma-Aldrich. Tween® 20 (Croda International PLC, Cowick Hall Snaith, UK) was purchased from Panreac. Iron standard solution was obtained from Acros Organics. Bis(sulfosuccinimidyl) suberate (BS³) was purchased from Pierce Biotechnology. Water (double processed tissue culture) used in all micro-supports synthesis and 14 kDa dialysis tubing celullose membrane were from Sigma. Mini-PROTEAN® TGX Stain-Free Gels 8-16% and Pro-Sieve® Color Protein Markers were purchased from Bio-Rad and Lonza respectively.

2.2 Synthesis of magnetic nuclei.

Magnetite nanoparticles were prepared following a previously described co-precipitation method [39]. Briefly, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ in the ratio 3:1 were dissolved in 18 mL of water and 6 mL of 30% $\text{NH}_3 \cdot \text{H}_2\text{O}$ was added drop wise under gentle stirring to form magnetic nuclei. After 20 minutes, magnetite nanoparticles were precipitated magnetically to remove the reactants and they were washed three times with water and finally they were suspended in 10 mL of water. To obtain polydopamine coating, 240 mg of dopamine were dissolved in 80 mL of 0.1 M borate buffer pH 8. Then, 76 mg of MNP, measured in terms of iron content, were added to the solution that was stirred for 30 minutes before separating coated magnetic nanoparticles from the eventually formed non-magnetic PDA particles by using a magnetic rack. PDA-coated magnetic nanoparticles (PDA-MNP) were washed three times with water and suspended in 10 mL of fresh water.

2.3 Synthesis of polysaccharide-stabilized micro-supports.

Micro-supports were obtained using a nanoemulsion method previously described in the work of De Matteis et al. [37] and slightly modified for the purposes of this work. 30 mg of PDA-MNP were added to 4 mL of water solution containing 6.8 mg Tween® 20. Then, the organic phase containing 4.3 mg Span® 85 and 20 mg oleic acid in 2 mL absolute ethanol was added to the aqueous phase under stirring. Nanoemulsion spontaneously and almost immediately formed. Then

0.25 mL of a 5 mg/mL chitosan solution in acetic acid 1% (v/v) or 1.25 mL of a 10 mg/mL alginate solution in water were added to stabilize the obtained nanoemulsion. In the last step of the synthesis, chitosan-coated nanoemulsion supports (CS) were added to 20 mL of 50 mM Na₂SO₄ under manual stirring. In the case of alginate-coated supports (ALG), they were washed twice with water before they were added to 20 mL of a 10% CaCl₂ solution. Both kind of micro-supports were washed three times with water and resuspended in 2 mL of fresh water.

2.4 Synthesis of polysaccharide external shell.

25 mg of ALG or 10 mg in the case of CS were resuspended in 6 mL 50 mM acetate buffer pH 5 under stirring. Then 0.65 mL of a 10 mg/mL sodium alginate solution or 0.25 mL of 5 mg/mL chitosan solution were added drop-wise respectively to the CS or ALG suspensions and keep stirring for 30 minutes.

Finally, alginate-externally coated micro-supports (ALG-CS) were washed twice with water and they were added to 20 mL of a 10 % CaCl₂ solution, while chitosan-externally coated micro-supports (CS-ALG) were added to 20 mL of 50 mM Na₂SO₄ under manual stirring. Both kind of double-coated micro-supports were washed three times with water and they were resuspended in 2 mL of fresh water.

2.5 Stabilization of chitosan magnetic micro-supports with BS3.

For the crosslinking of CS, 200 nmol of bis(sulfosuccinimidyl) suberate (BS³) were added drop-wise to a solution of 10 mg of micro-supports in 40 mL of 10 mM borate buffer pH 8.2 and they were kept under stirring for 30 min. Micro-supports were washed twice with water and resuspended in 2 mL.

2.6 Characterization of polysaccharide-coated magnetic micro-supports.

Iron content of micro-supports in water was obtained spectrophotometrically using a Varian Cary 50 UV/Vis spectrophotometer. Magnetic micro-supports were dissolved in a solution of nitric acid/hydrochloric acid. Then a solution of KSCN 0.75 M was used to form a complex with Fe(III) that absorb visible light at 484 nm. Iron concentration was obtained using a calibration with iron standard solution.

The encapsulation efficiency of MNP in polysaccharidic micro-supports was obtained calculating the ratio between the amount of iron encapsulated (recovered amount), measured dissolving the final material, and the amount of iron added during nanoemulsion formation (initial amount). The iron loading has been calculated from the ratio between the amount of iron recovered after encapsulation and the total weight of the final material obtained after freeze-drying.

Z Potential of supports has been determined by measuring the potential of a 0.05 mg/mL nanoparticle suspension in 1 mM KCl solution with a Plus Particle Size Analyzer (Brookhaven Instruments Corporation).

Fourier Transform Infrared Spectroscopy analysis was carried out in a JASCO FTIR—4100 Fourier transform infrared spectrometer in a frequency range of 600–4000 cm⁻¹ with 2 cm⁻¹ resolution and a scanning number of 32.

Samples were analyzed by means of Environmental Scanning Electron Microscopy (ESEM) in a QUANTA-FEG 250 microscope. The sample was analyzed without any previous treatment. The observation was carried out using a cooling stage (at approximately 1 °C) at humidity conditions of approximately 100%.

2.7 Stability assay.

The stability of the support under reaction conditions was evaluated by incubating 5 mg of each magnetic microsupport with phosphate buffer 10 mM pH 2.75 and measuring the release of dopamine during time.

2.8 Chloroperoxidase encapsulation and catalytic assay.

26,78 U of CPO (1 µL) were added to 0.25 mL of chitosan 5 mg/mL, before chitosan shell synthesis, depending on the material to be prepared, after nanoemulsion formation or after alginate first shell synthesis.

The encapsulation efficiency of the enzyme was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis (SDS-PAGE). Supernatants of encapsulation, as well as of the conditioning solution (phosphate buffer), were analyzed to quantify CPO content.

Supernatants from encapsulation process were dialyzed overnight using 14 kDa dialysis tubing cellulose membrane. All samples were freeze-dried in a Telstar Cryodos freeze-dryer and resuspended in a much smaller volume to be concentrated at a maximum theoretical concentration of up to 6 µL of enzyme per milliliter of solution.

Samples were denatured by boiling them in presence of a SDS/β-mercaptoethanol mixture before being loaded into the gel. Silver staining method was used to reveal the electrophoretic gels and band intensities were estimated by using ImageJ software. Calibration curve was performed by loading CPO samples at known concentrations (1-3 µL of enzyme per milliliter of solution) so the amount of enzyme in the supernatants was determined by comparison.

The chlorination of monochlorodimedone to dichlorodimedone was used as model reaction for chloroperoxidase catalytic assay. 10 mg of micro-supports were washed with 1 mL of 10 mM phosphate buffer pH 2.75. Micro-supports were separated from the buffered washing solution using a magnetic rack and the solution was recovered to evaluate the leaching of enzyme from the micro-supports.

Previously recovered micro-supports were mixed with 4 mL of buffered reaction mixture containing 3.6 mL of $3.19 \cdot 10^{-4}$ M MCD solution prepared using $3.19 \cdot 10^{-2}$ M KCl in phosphate buffer pH 2.9 and 0.4 mL of 4.6 mM H₂O₂ and kept under stirring for 5 minutes. After each reaction cycle, micro-supports were separated from the mixture using a magnetic rack.

The supernatants were analyzed spectrophotometrically and the reaction yield was determined in terms of monochlorodimedone absorbance decrease and calculated as ratio percentages between the reaction mixture absorbance before and after a reaction cycle of 5 minutes ($\text{Abs}_{\text{final}}/\text{Abs}_{\text{initial}} \times 100$). If conversion was complete, the catalytic system was recycled directly (without any washing treatment) by adding a freshly prepared reaction mixture.

In the case of an incomplete conversion after 5 minutes, the reaction mixture and the micro-supports were left to react again during a longer time.

Magnetic micro-supports without immobilized enzyme were also tested as negative controls in terms of catalytic activity using the same reaction conditions and also to discard any absorption of the substrate on micro-supports. Freshly prepared micro-supports were used in all experiments. They were washed with water, resuspended and kept at 4.0°C when experiment length did not allow their continuous use.

All sets of experiments were reproduced several times under identical operating conditions in order to increase the accuracy of the findings.

2.9 Leaching assay.

To quantify the possible leaching of the enzyme, the activity of the washing supernatants was assessed mixing 1 mL of the supernatant of the coating and entrapment process with 9 mL of the reaction mix which contained 0.4 mL of H₂O₂ 4.6 mM and 8.6 mL of a 1.15·10⁻⁴ M MCD/1.15·10⁻² M KCl solution in phosphate buffer pH 2.9. The activity of released CPO was determined as ratio percentages between the reaction mixture absorbance at 278 nm before and after a reaction cycle of 5 minutes.

3. Results and Discussion

3.1 Magnetic micro-supports synthesis

Magnetic nanoparticles represent a very interesting building block for the development of nanoscaled supports for enzyme immobilization. Iron oxide nanoparticles, obtained by co-precipitation method, were chosen since they can be very easily produced in high amounts and they offer the possibility to be coated with the most useful materials for enzyme immobilization.

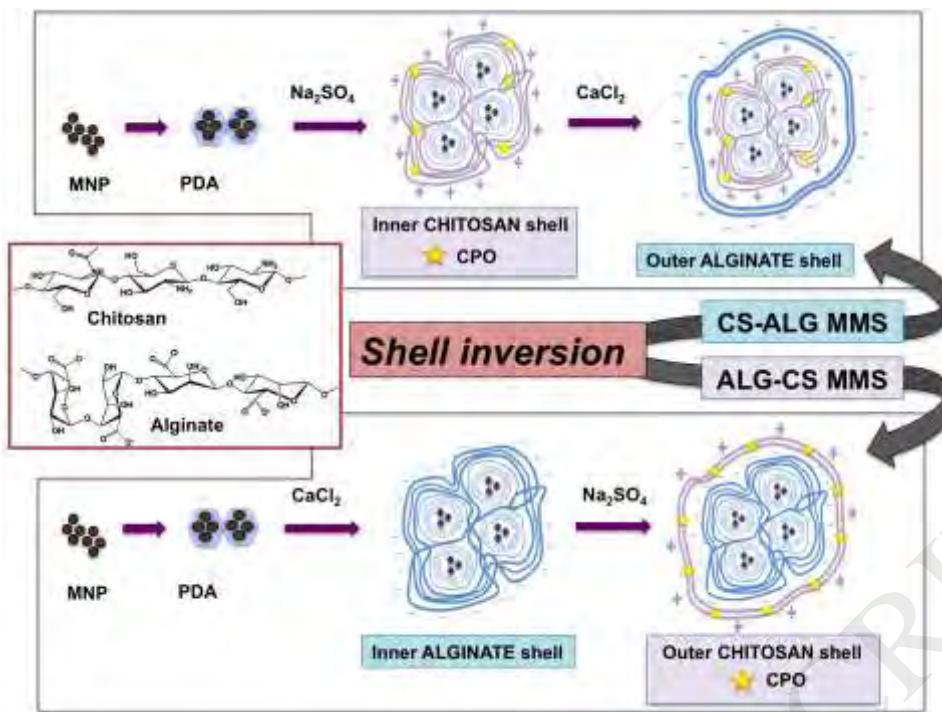
More concretely, superparamagnetic iron oxide nanoparticles with a diameter between 5 and 10 nm were synthesized using a method previously optimized [39] through which a water-stable colloidal suspension was obtained. A thin shell of polydopamine was formed around the magnetic nuclei through spontaneous oxidative polymerization of dopamine under slightly basic pH value to passivate the surface of iron oxide nanoparticles, preventing any deterioration of their crystalline nature and so the eventual loss of magnetic properties. Dopamine was added to the nanoparticle suspension to allow firstly the coordination on iron oxide surface through catechol group and subsequently its polymerization to form an organic thin layer of polydopamine surrounding the inorganic nuclei. Polydopamine was chosen to favor the following encapsulation of magnetic nanoparticles in a nanoemulsion-based system by improving their affinity. In fact, previous studies on the encapsulation of naked nanoparticles using the same nanoemulsion strategy lead to very unsatisfactory results in terms of encapsulation efficiency and homogeneity of the whole system obtained (data not

shown). In consequence of polydopamine coating, a significant improvement of the affinity of nanoparticles for the nanoemulsion components was observed. Very homogeneous magnetic nanoemulsion cores were obtained using a method based on a previously optimized synthesis [37].

The stabilization of these cores was obtained using natural polysaccharides, sodium alginate and chitosan, that formed a nanohydrogel shell around the magnetic nanoemulsion nuclei. These polymers were chosen mostly for their biocompatibility and availability but also for their versatility to form nanoparticles and coatings [20,40]. In fact, the coating of magnetic nanoparticles with polymers can be carried out using different strategies. Moreover, different kinds of interactions and both synthetic and natural polymers can be used. Although synthetic polymers can be tailored to meet specific properties, natural polymers as polysaccharides have the advantage of being abundant in nature, they are biodegradable, cheaper and present greener extraction/production processes [12].

After a first interaction of the polymer with the cores to favor their adsorption on micelle aggregates, the mixture was put in contact with a Na_2SO_4 and CaCl_2 solution, respectively in the case of chitosan and sodium alginate, to produce the interaction between polymer chains necessary to obtain the gelification of the polymeric coating.

Apart from the iron oxide magnetic nuclei, the organic nature of the support makes it possibly sensitive to reaction conditions. The search for the most robust coating was necessary to avoid any loss of support properties during the recycling process of the catalytic nanosystem. So, in order to select the most efficient system for enzyme immobilization, different strategies were developed, involving different combinations of the two polysaccharides on the surface of the magnetic nanoemulsion-based supports. The first approach consisted in the covalent crosslinking of chitosan network containing the immobilized CPO. Also a double, mixed polymer layer was used as strategy to reinforce the support. In particular, chitosan and sodium alginate were used in two different combinations (inner chitosan shell, outer alginate shell or the opposite combination) as reported in Scheme 1. Finally, five different magnetic supports were obtained and they undergo to full characterization and stability assays to assess their performances in terms of material properties and enzyme reusability. Independently on the coating strategy, chitosan was chosen to create an optimal microenvironment for enzyme entrapment thanks to its superior stabilizing effect on CPO, as observed in our previous work [36]. So, in all tested combinations of coatings, enzyme was always entrapped in chitosan shell.



Scheme 1. General description of polysaccharide shell combinations used to stabilize the nanoemulsion magnetic cores.

3.2 Magnetic micro-supports characterization

The modifications on the shell of the magnetic micro-supports were studied and confirmed using different techniques for the physicochemical characterization of the material.

In particular, FTIR and Z-Potential analysis were used to confirm the successful coating of magnetic cores with the different polymers. In Figure 1 FTIR spectra of the optimized supports and also of the magnetic nanoparticles coated with only polydopamine are reported.

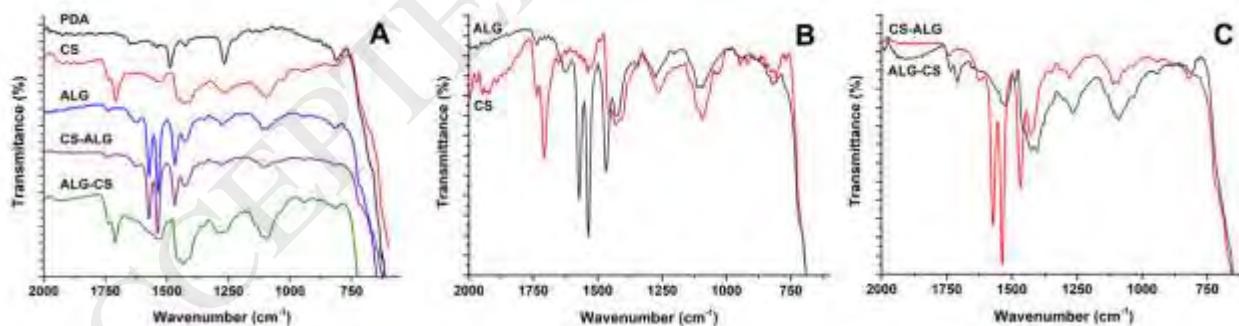


Figure 1. FTIR analysis of magnetic micro-supports: A) comparison of FTIR spectra of all micro-supports B) comparison between single shell micro-supports (ALG and CS); C) comparison between double shell micro-supports (CS-ALG and ALG-CS).

FTIR spectra were registered after each step of the coating to describe the modification on the surface of the different supports. In Figure 1A all spectra are reported to allow their comparison while in Figure 1B and 1C the comparison between single shell systems and the double-shell ones are reported for a more accurate description. From Figure 1B it

can be observed that the method used to obtain the stabilization of the nanoemulsion cores by covering them with polysaccharides is very effective since the peaks corresponding to most representing vibrations of the polymers structure can be recognized in both cases. In particular, characteristic absorption bands of chitosan at 1648 and 1540 cm^{-1} could be attributed to the stretching of C-O (amide I) and N-H bending (amide II), respectively. The carbonyl vibration of the acetic acid used to solubilize the polymer is noticeable at 1705 cm^{-1} . Moreover, absorption bands, characteristic of the polysaccharide structure, appear at 1100 cm^{-1} (stretching of C-O-C glycosidic bridge) and 1030 cm^{-1} (skeletal vibrations involving the C-O stretch). In the case of ALG single shell micro-supports, the characteristic absorption bands related to the asymmetric and symmetric stretching of -COO⁻ groups are shifted to 1536 and 1465 cm^{-1} with respect to the pure polymer (1595 and 1405 cm^{-1}). These shifts could be due to the interactions between the polymer and the components of the nanoemulsion or with the Ca⁺² ions used for crosslinking. In the case of double-shell supports, it can be concluded from the analysis reported in Figure 1A and 1C that only the signal corresponding to the external shell can be detected by FTIR, indicating a masking effect of the second shell possibly due to the interactions established between the two layers.

With the same purpose of describing the changes on micro-support surface, especially in the case of single and double layer coatings, Z-potential analysis was also carried out. It resulted from the analysis that the potential of the surface of the obtained magnetic supports clearly reflected the nature or combination of the polysaccharides used. Results are reported in Figure 2.

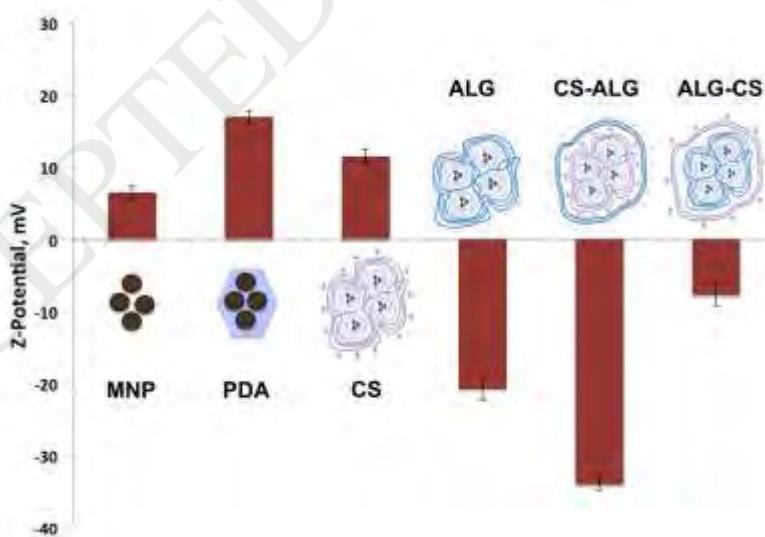


Figure 2. Z-potential analysis of magnetic micro-supports with and without polymeric shells.

As expected, chitosan and alginate single shells lead respectively to a positive and negative potential due to the presence of their amine and carboxylic groups. The outer alginate shell on chitosan surface leads to a high negative potential, probably due to the strong effect of alginate hydrogel that seems to completely mask the one exerted by the inner chitosan layer. In the case of the reverted combination, in presence of an outer chitosan shell, the potential was still slightly negative. In this case the effect produced on the surface potential by the presence of chitosan polymer was not strong enough to completely mask the alginate influence. The potential in this case is not as positive as the one of chitosan single shell. Nevertheless the presence of the outer chitosan shell can be clearly appreciated as well as the opposite contribution of the alginate inner layer still evident.

The morphological characterization of the materials produced in this work has been carried out using the Transmission Electron Microscopy (TEM) as well as the Environmental Scanning Electron Microscopy (ESEM) in the case of the magnetic micro-supports that required especially soft conditions during the analysis since they showed to be very sensitive to conventional electron microscopy operative conditions. In Figure 3, images referring to TEM analysis of iron oxide nanoparticles naked (A) and coated with polydopamine (B) are reported, together with ESEM images referring to CS-ALG and ALG-CS magnetic micro-supports (Figure 3 C and D respectively).

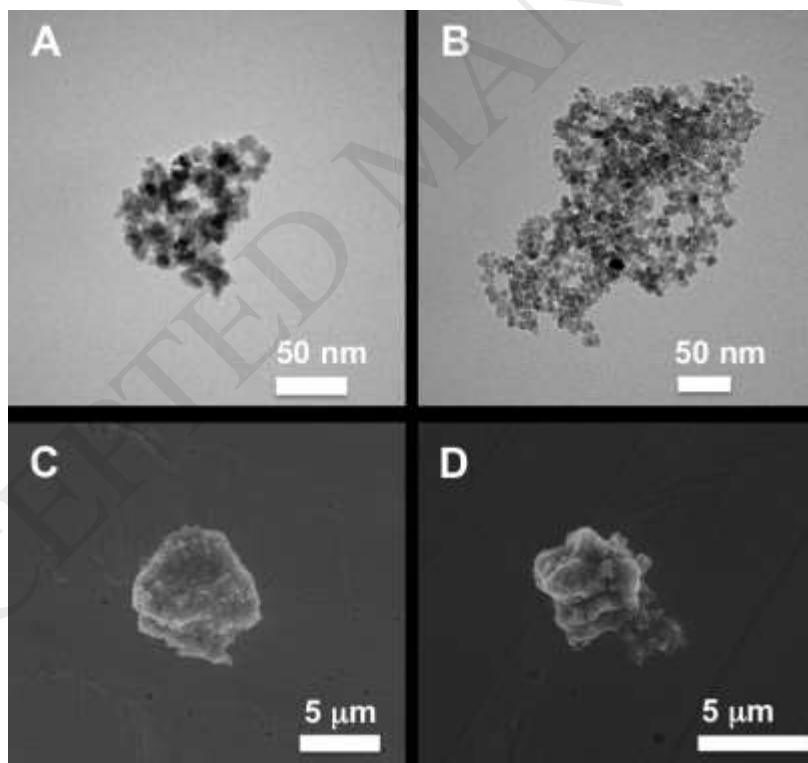


Figure 3. A) TEM image of iron oxide nanoparticles; B) TEM image of polydopamine-coated iron oxide nanoparticles; C) ESEM image of CS-ALG magnetic micro-support and D) ESEM image of ALG-CS magnetic micro-support.

It is possible to appreciate from the images that naked magnetic nanoparticles, as well as the polydopamine coated ones, have a size around 5-10 nm and although they appear aggregated in larger clusters, this effect has to be addressed

to the sample preparation method and especially to the drying effect of samples in water suspension. In fact, both kind of nanoparticles are very stable in water suspension. After their encapsulation using the nanoemulsion method and the following polymer coating, it is hypothesized that nanoemulsion cores are coated in bigger aggregates surrounded by the polymeric layer. This aggregation leaded to the formation of micro-supports with a size of few microns and this effect seemed to be independent from the combination of polymeric coatings, as shown in Figure 3 C and D.

3.3 Functional stability of magnetic micro-supports

The chemical stability of the developed micro-supports in the reaction medium was measured in terms of dopamine release during time when the supports were put in contact with phosphate buffer used in the enzymatic reaction. In fact, it is reported that polydopamine coating is not stable under acidic conditions [41] and any release of polydopamine and/or dopamine can be measured spectrophotometrically. It should be specified that identical results were obtained using the complete reaction medium (data not shown), clearly confirming that the degrading effect has to be completely addressed to the buffer. Stability results obtained using the developed magnetic micro-supports without enzyme are reported in Figure 4. The point at time 0 refers to incubation in the aqueous storage medium in which no release of dopamine/polydopamine was observed.

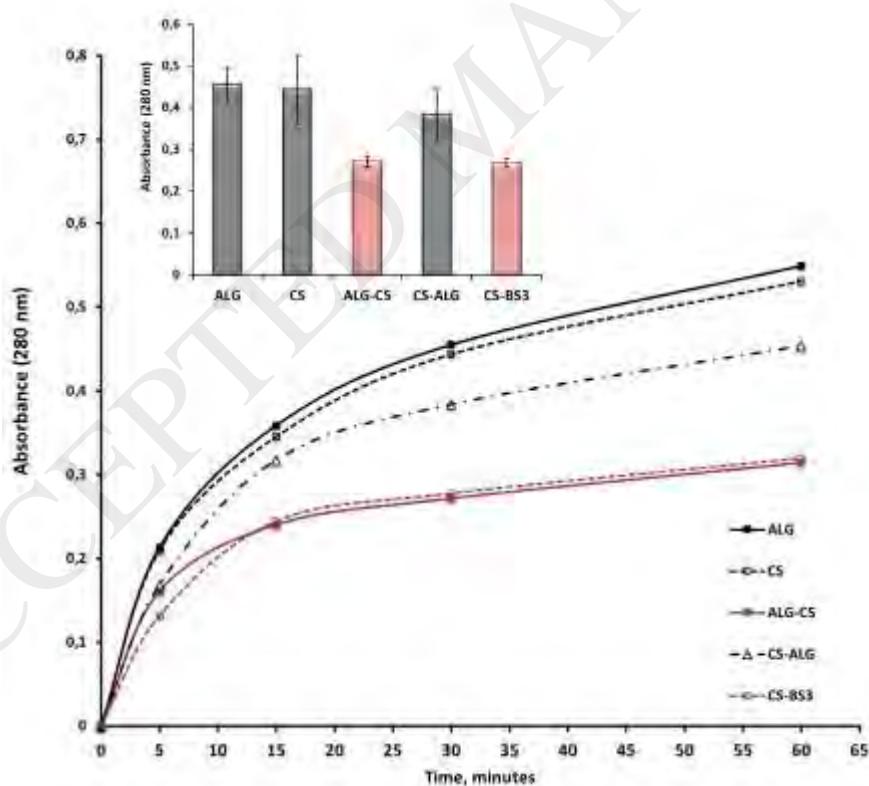


Figure 4. Stability assay of magnetic micro-supports. Dopamine/polydopamine release in phosphate buffer 10 mM pH 2.75. Inset: data referring to 30 minutes incubation.

It was found that a single polymer shell was not robust enough to protect the support from the release of chemical components (especially dopamine on iron oxide surface) in presence of acidic phosphate buffer in the reaction medium. In fact, in the case of chitosan, the acidic pH of the medium leads to the swelling of the polymer shell, while in the case of alginate, the presence of sodium phosphate salt leads to the wash out of Ca^{2+} ions and to the consequent degradation of hydrogel network [42,43]. Both effects cause the loosening of the polymeric network.

A significant decrease in polymer swelling was obtained through the rigidification of the chitosan shell by the reaction of the homobifunctional crosslinker bis(sulfosuccinimidyl)suberate (BS3) that covalently linked amino groups on polysaccharide chains. A similar improvement was obtained using the support with a sodium alginate internal shell and an outer chitosan layer. On the other hand, only a very small improvement was observed using the inverted shell combination (that means chitosan internal shell and sodium alginate outer layer), which behaved like the support with a single chitosan shell.

After enzyme immobilization, the encapsulation efficiency and the enzyme leaching were measured by SDS-PAGE analysis (Figure 5).

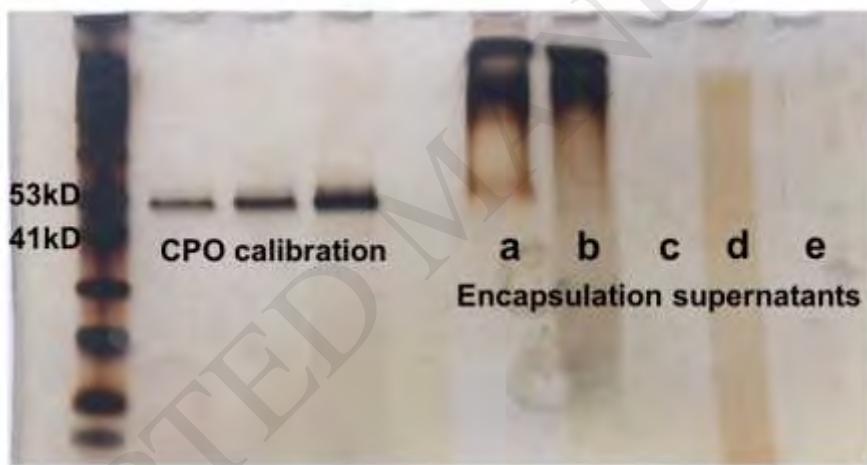
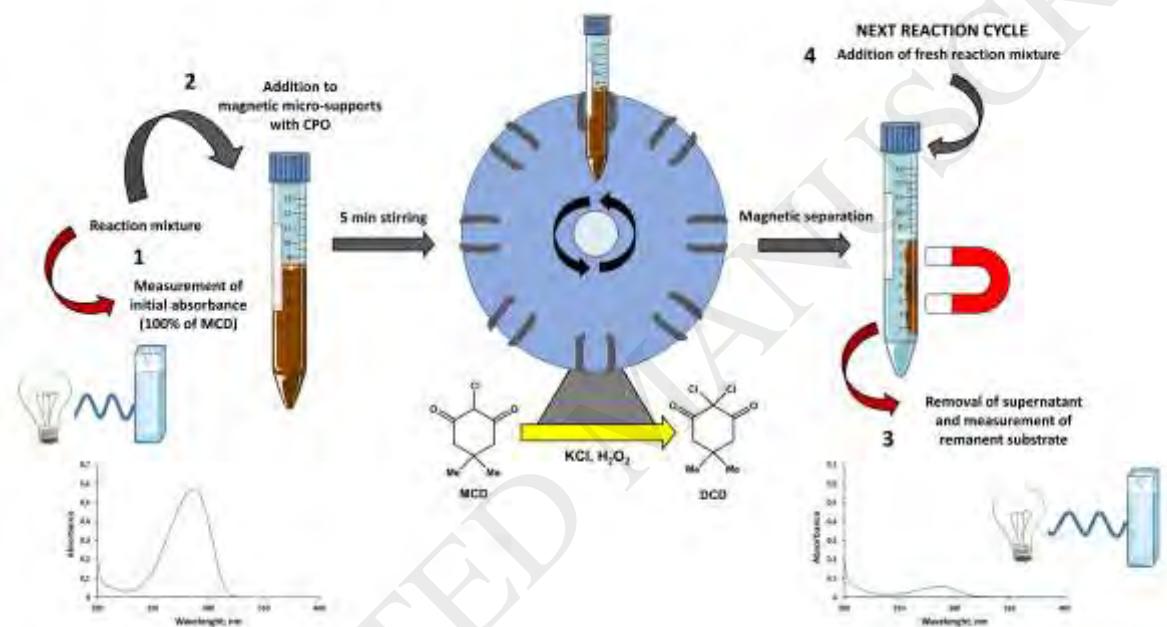


Figure 5. SDS-PAGE analysis of encapsulation supernatants: a) CS supernatant, b) ALG-CS MMS supernatant, c) CS-BS3 supernatantMMS, d) CS-ALG washing solution before gelation and e) CS-ALG supernatant of gelation.

The presence of free CPO was investigated in both supernatants of the encapsulation process and in buffered solutions used for the conditioning of the micro-supports before the first reaction cycle (data not showed).

After encapsulation of the enzyme in chitosan shell, when it is added directly on nanoemulsion core, 8% of total CPO added was detected in the supernatant. No further leaching was observed after the following coating with alginate or crosslinking with BS3, as well as in the inverted coating on alginate inner shell, taking into account a detection limit of 5%

The developed magnetic micro-supports with encapsulated enzyme were used to test the activity and reusability of the catalytic systems for monochlorodimedone conversion to dichlorodimedone. Reaction conditions, in terms of enzyme/substrate ratio and concentrations, were the same that the authors used in previous studies [36]. The conversion yield was calculated measuring the decrease in the absorbance of monochlorodimedone after 5 minutes of reaction. If a conversion of 100% was obtained, micro-supports were recovered and put in contact with a fresh reaction mixture to be reused in a second reaction cycle. It has been observed that when supports were washed with buffered solution before a second reaction cycle, a slightly lower reusability efficiency was obtained in terms of number of consecutive cycles of reaction (data not shown). A description of the whole process (catalytic assay and recycling) is reported in Scheme 2.



Scheme 2. General description of catalytic assay and recycling procedure.

Chloroperoxidase encapsulated in the support consisting of a double layer with an outer shell of sodium alginate, as well as in the support coated with chitosan and crosslinked with BS3, showed almost no activity even at the first reaction cycle (Figure 6A). The lack of activity of entrapped CPO could be addressed to poor substrate diffusion through the polymeric shell in the first case or to the any involvement of the polypeptide structure in the crosslinking in the case of BS3-treated supports. In fact, rigidification of the protein structure through its covalent linking to the polymer network and the consequent decrease in the flexibility or the distortion of the active site would probably result in the inactivation of the catalyst.

On the other hand, the enzyme immobilized in the supports with a chitosan single shell and the double layer-support with outer chitosan shell maintained its activity during several reaction cycles. Data concerning the reusability of these two catalytic systems is reported in Figure 6B.

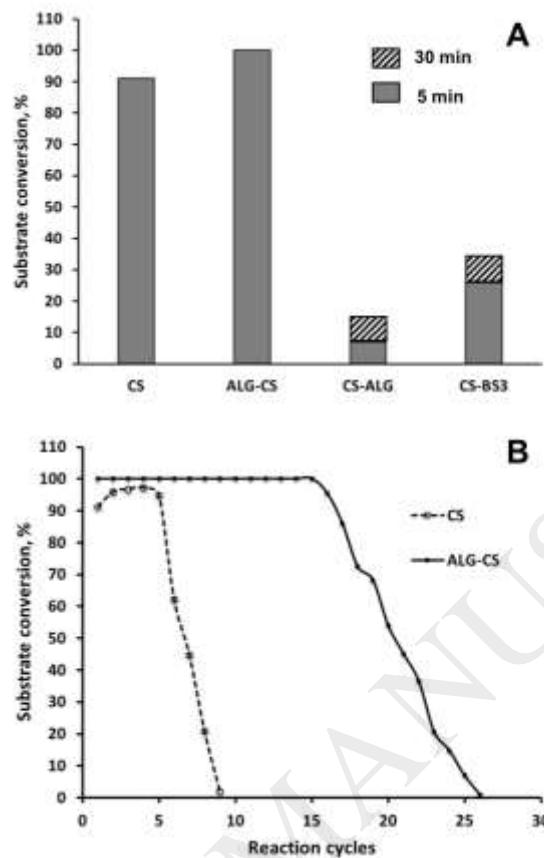


Figure 6. A) Reaction yields after the first reaction cycle. B) Reusability of chitosan-coated (CS) and double shell coated micro-supports with alginate inner shell (ALG-CS).

In the case of the support with chitosan single shell, the catalytic activity dramatically decreased after 5 reaction cycles. The loss in enzymatic activity could be addressed in this case to the progressive leaching of the enzyme from the support. The presence of free enzyme in the washing steps of encapsulation and in the buffer used for the conditioning of the supports just before the first reaction cycle was detected by adding monochlorodimedone and measuring the conversion (data not shown). Only in the case of micro-supports with a chitosan single shell the leaching of the catalyst was detected and a 77% and 97% of conversion were measured respectively in the encapsulation medium and conditioning medium after 15 minutes of incubation with the reaction mixture. Data were in agreement with the encapsulation efficiency of the enzyme measured by SDS-PAGE analysis.

The catalytic system based on an inner alginate shell and a chitosan outer shell with entrapped CPO showed a remarkably higher reusability. The better chemical stability of this carrier and probably the optimal permeability of the shell containing the catalyst, lead to a reusability of more than 20 reaction cycles. The catalytic system was able to

convert the substrate with a 100% of efficiency during 16 consecutive reaction cycles. The following cycles were carried out with partial efficiency and longer reaction times but the system still presented a 23% of conversion at cycle 23. The reusability obtained with CPO entrapped in ALG-CS supports was comparable or even better than that reported in literature for other magnetic support with immobilized CPO (Table 1) and results clearly confirmed that chitosan-based materials represent a very suitable microenvironment for chloroperoxidase immobilization and reuse. The optimization of the support properties carried out in this study to obtain a magnetic support with a stabilizing polymer shell demonstrated that supported catalysts with superior properties could be obtained developing smart materials that gather properties of their different components.

Table 1. Comparison of reuse efficiency of CPO immobilized on different supports.

Support	Immobilization technique	Reuse efficiency	Ref.
Mesoporous silica matrix	Entrapment by electrostatic interactions	20 cycles with 61% residual activity	[25]
Magnetic nanoparticles	LBL assembly through avidin-biotin affinity binding	20 cycles with 62% residual activity	[26]
Magnetic iron oxide NP with polymer shell	Covalent	12 cycles without loss in activity	[27]
Au@Fe ₃ O ₄ nanoparticles	LBL assembly through avidin-biotin affinity binding	12 cycles with more than 62 % residual activity	[28]
TiO ₂ mesoporous thin films	Physical adsorption	7 cycles without loss in activity	[29]
ZnO nanowires/macroporous SiO ₂ composite	Cross-linking	12 cycles with 81% residual activity	[31]
Graphene oxide nanosheets	Site-specific interactions with concavalin A	8 cycles with 52% residual activity in the first run	[34]
PEG-doped silica matrix	Entrapment	4 cycles without loss in activity	[35]
Polysaccharide-silica matrices	Entrapment	18 consecutive cycles	[36]

4. Conclusions

Enzyme immobilization in magnetic supports offers many advantages for the reusability of the catalytic system. The use of these supports allows to easily separate the catalyst from the reaction medium and to reuse it during several reaction cycles, highly improving the cost-effectiveness of synthetic processes.

In this work, a combined strategy of using magnetic nanoparticle-based supports with polysaccharide coatings was chosen to have the additional advantage due to the already known stabilizing effect on chloroperoxidase of polysaccharides as alginate and especially chitosan.

The possibility of combining different polymers to obtain multi polysaccharide shells have been successfully explored together with the covalent crosslinking of chitosan for the improvement of polymer shell rigidity. Interesting differences in micro-supports chemical stability under reaction conditions have been observed. Among the most stable magnetic

supports obtained, the one consisting in an alginate inner shell and a chitosan outer shell, containing the entrapped enzyme, showed the best reusability of the catalytic system, allowing 25 consecutive cycles of model substrate conversion. This result indicates that the catalytic properties of the enzyme are preserved by protecting it from the effect of denaturing agents and through physical stabilization of the protein in the microenvironment of the support as expected.

On the basis of the interesting results obtained, future works will be devoted to test the developed system for the catalysis of more interesting reactions catalyzed by chloroperoxidase for synthesis of fine-chemicals.

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