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Preparation of submicrometer monodispersed magnetic silica particles using a novel water in oil microemulsion: properties and application for enzyme immobilization

Maria Victoria Tuttolomondo · Maria Emilia Villanueva ·
Gisela Solange Alvarez · Martín Federico Desimone ·
Luis Eduardo Díaz

Abstract The synthesis of monodispersed magnetic silica nanoparticles (MSN) is described using a water-in-oil reverse microemulsion system that does not require the use of co-surfactants. Sodium silicate, Tween 20 as a neutral surfactant and 1-butanol as the organic phase were used. There are several advantages of the proposed method including a saturation magnetization value of 10 emu/g for the particles obtained, uniformity of size and that they are easily functionalized to bind urease covalently. Moreover, the intra-day, inter-day and long-term stability results confirm that the procedure was successful and the enzyme-linked MSNs were stable over repeated uses and storage retaining more than 75 % activity after 4 months.

Keywords Enzyme immobilization · Magnetic nanoparticles · Silica nanoparticles · Urease

M. V. Tuttolomondo · M. E. Villanueva ·
G. S. Alvarez · M. F. Desimone (✉) · L. E. Díaz
IQUIMEFA-CONICET, Facultad de Farmacia y
Bioquímica, Universidad de Buenos Aires, Junín
956 Piso 3°, 1113 Buenos Aires, Argentina
e-mail: desimone@ffyb.uba.ar

Introduction

The preparation of silica nanoparticles with controlled morphology is receiving increasing attention due to their potential applications as catalyst supports, sorbents and compartments for the storage and release of molecules (Desimone et al. 2010; Foglia et al. 2011). Inverse microemulsions have been widely used as templates for nanoparticle synthesis (Cellesi and Tirelli 2006; Lou et al. 2008; Sierra and Guth 1999; Sierra et al. 2000). These thermodynamically-stable emulsions act as reactors for hosting the polymerization reaction and also act as steric barriers that inhibits the polymerization among reactive species between different droplets during the reaction period (Chang and Fogler 1997; Lopez-Quintela 2003). The preparation of mesoporous silica using non-ionic surfactants (neutral route) has important advantages over electrostatic routes, because of the easy removal of the surfactant by solvent extraction and the tendency to produce materials with thicker walls and smaller particle sizes (Sierra and Guth 1999).

Functionalized supraparamagnetic iron oxide nanoparticles (SPIONs) have also received worldwide interest. Silica-coated magnetic nanoparticles provide many surface reactive groups, substantially via silanol groups, to be directly employed in subsequent surface grafting (Rostamian et al. 2011). Especially, amino-silane modified magnetic iron oxide/silica core/shell nanoparticles are magnetic materials that can be successfully applied in enzyme immobilization protocols due to their ease of

preparation and applicability (Atia et al. 2009; Schultz et al. 2007; Wang et al. 2010). Moreover, enzyme immobilization on inert magnetic carriers has the potential advantages of significant cost savings, facilitating enzyme recycling through multiple reaction cycles, improving stability or resistance to shear or inhibitory compound inactivation (Hsieh et al. 2009; Mateo et al. 2007; Ó'Fágáin 2003; Sheldon 2007).

In this study, we report the synthesis of monodispersed MSNs, using a water-in-oil (W/O) reverse microemulsion system that does not require the use of cosurfactants. We propose the use of a sodium silicate solution, Tween 20 as a neutral surfactant and 1-butanol as the organic phase. To the best of our knowledge, this system has not been reported before to obtain enzyme linked monodisperse MSN. Moreover, the characterization of the magnetic particles obtained and the results of immobilization of urease onto the magnetic silica particles, as well as its intraday and interday reutilization stability are reported. This system has proven to be advantageous over previously reported ones, especially for the high levels of magnetization retained, uniformity of the particles and biocompatible enzyme immobilization.

Materials and methods

Chemicals

3-Aminopropyltriethoxysilane (APTES) and urease (EC 3.5.1.5) from jack bean (54300 U/g) were from Sigma. Urea colorimetric detection kit was from Weiner Lab. (Argentina). All chemicals were used as received. Deionized water was used throughout.

Synthesis of ferrofluid

The synthesis of the ferrofluid was performed using a co-precipitation method (Goodarzi et al. 2003). $FeCl_2 \cdot 4H_2O$ and $FeCl_3 \cdot 6H_2O$ were mixed in equal molar proportions in deionized water and then added dropwise to a 1 M NaOH at room temperature. Once the brown-black ferrofluid precipitate was obtained, it was washed with deionized water until a neutral pH was reached. The magnetic fluid was kept in deionized water until use. The solid content was 0.01 g/ml.

Synthesis of MSN

An inverse microemulsion composed of 1-butanol (71.6 g) and Tween 20 (14.3 g) as the organic phase and 15 ml sodium silicate solution (2.2×10^{-2} g/ml) mixed with 0.01 g/ml ferrofluid as the aqueous phase were used. The aqueous phase was added dropwise into the organic phase with constant vigorous agitation until small grayish particles began to precipitate. The MSNs were left overnight and washed several times with acetone to remove the remaining organic components. Finally, the MSNs were dry and stored until use.

Grafting of the MSN

MSNs were activated by an ultrasonic mix with 0.1 M NaOH for 15 min. Afterwards, they were washed twice with deionized water, once with acetone/water 1:1 (v/v) and finally once with acetone. The activated particles were grafted with 5 % APTES in acetone for 90 min at 37 °C with constant stirring (Hartono et al. 2010; Wu et al. 2006). After the reaction was over, the particles were washed twice with acetone, once with acetone/water (1:1 v/v) and once with 0.5 M phosphate buffer, pH 7.4. Once the washes were completed, particles were treated with glutaraldehyde in phosphate buffer 0.5 M, pH 7.4 for 60 min at 37 °C with constant stirring. To determine whether glutaraldehyde concentration influenced the final enzyme performance, different glutaraldehyde concentrations (from 0.025 to 0.25 %) were evaluated. MSNs were then washed three times with phosphate buffer and left to react with the enzyme (0.5 mg/ml) overnight at 37 °C with constant stirring.

After being washed three times with PBS to remove unbound enzyme, the unreacted aldehyde groups of the enzyme functionalized nanoparticles (MSNs-E) were blocked with 0.1 % monoethanolamine in buffer at 37 °C for 30 min. Finally, the particles were washed three times with phosphate buffer, resuspended in buffer and left at 4 °C until use.

Characterization of the nanoparticles

The average size distribution was measured with DLS (Zetasizer Nano-Zs, Malvern Instruments, Worcestershire, UK) provided with a He–Ne (633 nm) laser and a digital correlator ZEN3600, at 25 and 37 °C.

Measurements were conducted at a scattering angle of $\theta = 173°$ to the incident beam. Samples were analyzed using a scanning electron microscope (SEM). Elemental analyses were carried out by using an EDAX analyzer.

Urease activity and kinetic parameters

Urease activity was assessed using the indophenol method (see Desimone et al. 2008) and measuring the blue indophenol at 540 nm. Free urease and MSNs-E were incubated with an urea solution in 50 mM phosphate buffer pH 7, and their activities measured at 25 °C. Other aliquots of the MSNs-E suspension were added to samples and incubated with various increasing urea concentrations for 15 min at 25 °C. To evaluate the K_m and V_{max} of the system, the Lineweaver–Burk plot for the Mikaelis–Menten equation was used (see Lai and Tabatabai 1992). All enzyme assays were run in triplicate and average rates were calculated. Data are means $\pm$ SD. The differences were analyzed using unpaired t test where $p < 0.05$ was considered significant.

Recovery and stability assays

MSNs-E were suspended and incubated with urea solutions for 15 min at 25 °C. After incubation was completed, a magnet was used to separate the MSNs-E. The supernatant was collected and was used for ammonium determination by the indophenol method. MSNs-E were washed with phosphate buffer three times and employed again in the following assays. This procedure was repeated three times a day during 5 consecutive days in order to evaluate the stability of the system. MSNs-E were tested under the same conditions of the previous assay after three months storage at 4 °C.

Results

Magnetic properties of the ferrofluid and MSNs-E are shown in Fig. 1. The absence of hysteresis in both systems indicates that they exhibit superparamagnetic characteristics. Moreover, both remanence and coercivity are zero. The ferrofluid presented the highest magnetization value with a saturation value of 46 emu/g. The MSNs-E presented a saturation magnetization

value of 10 emu/g. This difference can be attributed to the presence of the grafted layer (i.e.: silica, glutaraldehyde and enzyme layers).

The optimum amount of glutaraldehyde was determined by SEM and enzyme activity measurements. Figure 2 shows the influence of glutaraldehyde concentration on the immobilized enzyme activity obtained. As the glutaraldehyde concentration increased, so did the enzyme activity because more enzyme can be covalently linked. However, when the glutaraldehyde concentration reached 0.25 %, the enzyme activity drastically decreased probably because of cross-linking between glutaraldehyde molecules, leaving less available aldehyde groups for enzyme attachment and thus diminishing the reactive beads' surface. SEM images of the nanoparticles grafted with different concentration of glutaraldehyde further confirmed these results (Supplementary Fig. 1). These results are in agreement with previously reported work (Chen and Chiu 1999; Liang et al. 2005). These results lead us to conclude that the optimum amount of glutaraldehyde to graft the MSNs is 0.1 %. Varying the amount of APTES, monoethanolamine and initial enzyme concentration in ± 20 % did not show significant variations by the results (data not shown).

Magnetic nanoparticles consist mostly of iron (22 %) and oxygen (40 %) (Fig. 3). The MSNs are composed mainly of oxygen (66 %) and silicon (18 %), while iron is detected in a very low level which is in close agreement with the iron/silica core/shell structure (Fig. 3b). Finally, the amount of iron
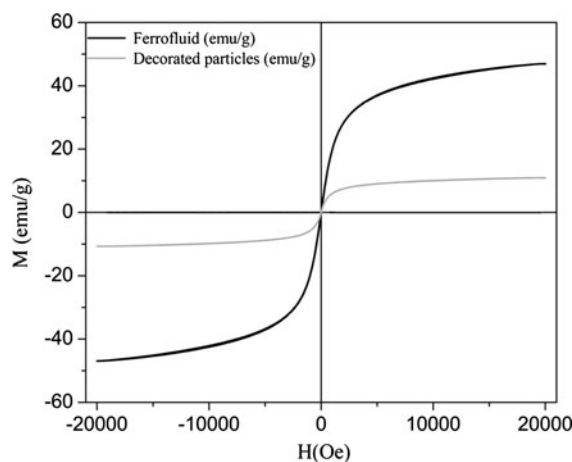

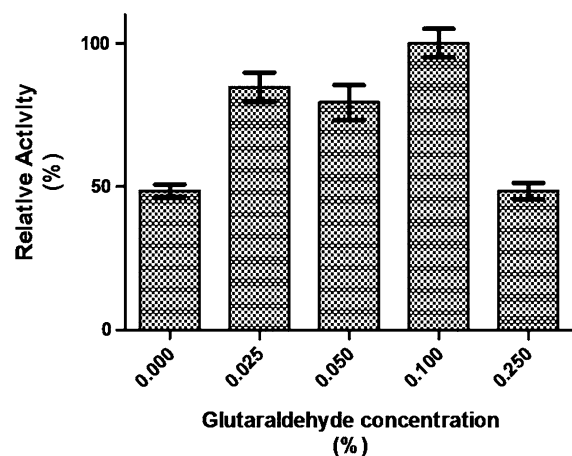**Fig. 1** Magnetic properties of the ferrofluid (*black*) and MSNs-E (*blue*)

and silicon detected diminish due to the formation of a new layer consisting mostly of carbon (15 %), oxygen (60 %) and silicon (10 %), provided this by the addition of APTES, glutaraldehyde and the enzyme (Fig. 3c).

Light-scattering results show that the mean diameter of the decorated nanoparticles is 467 nm which, when compared to the size of the nude nanoparticles (206 nm) suggests that the decoration layer (APTES, glutaraldehyde, urease, monoethanolamine) is, on average, 130 nm thick. Urease is an hexamer with a molecular weight of 545 kDa which explains the differences in the diameters of the nanoparticles.

The intra-day (three replicate assays) and inter-day (5 days with three replicate assays each time) repeatability were calculated by subjecting the same MSNs-E to repeated assays. Figure 4a shows that the intra-day enzyme activity loss is lower than 5 %. Meanwhile, the inter-day assay results indicate that near 80 % of enzyme activity is retained (Fig. 4b). Finally, the long-term stability result indicates that the MSNs-E are stable over 4 months when stored at 4 °C (Fig. 4c). Indeed the activity after this time is higher than 75 % of the initial activity, suggesting that the MSNs-E are both robust and stable.

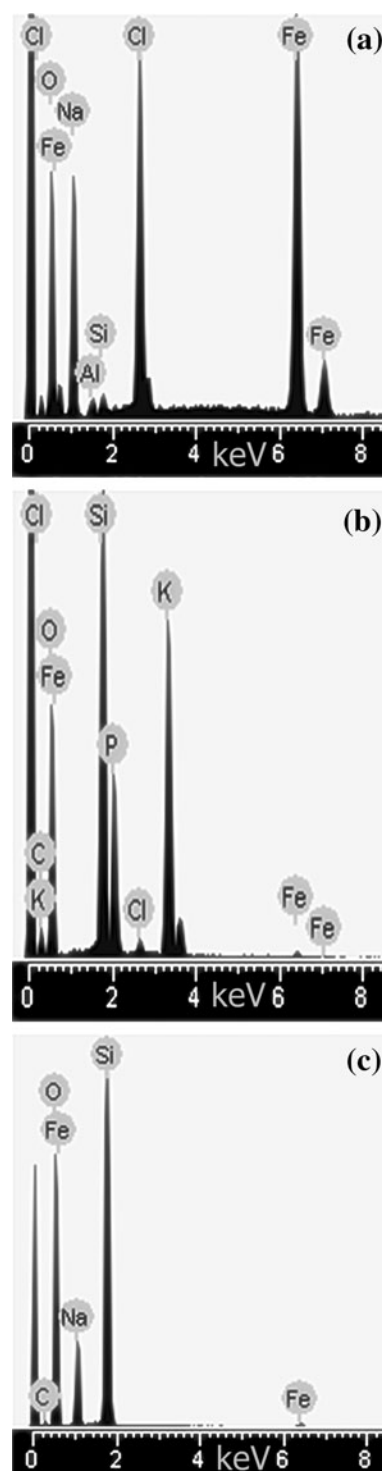To study the activity of the MSNs-E in real samples, different parameters have been changed in

Fig. 3 EDAX analysis of: **a** ferrofluid; **b** iron/silica core/shell nanoparticles and **c** MSNs-E
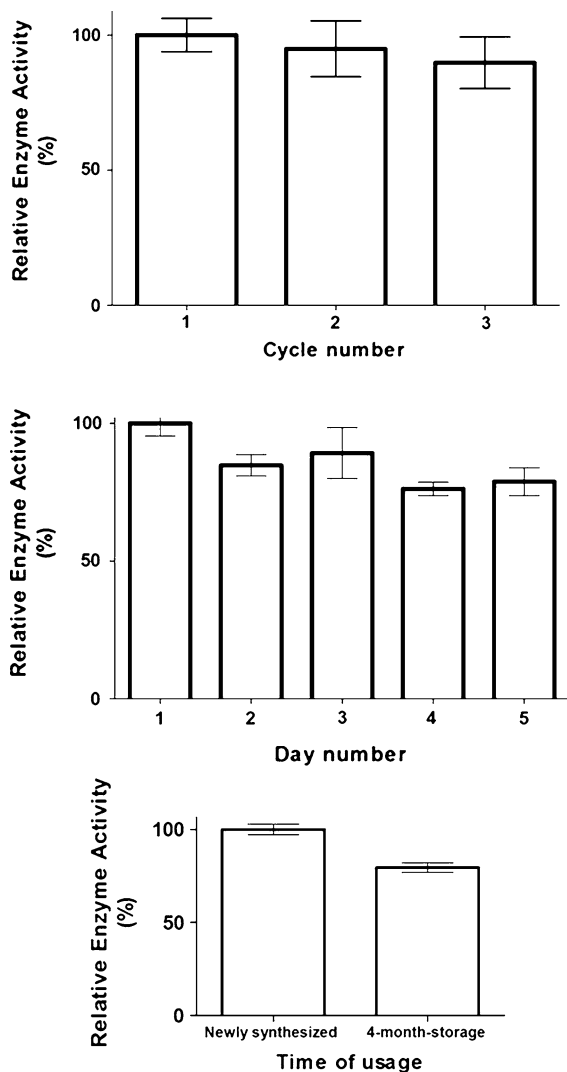
Fig. 4 Enzyme activity measurements: **a** Intraday repeatability (three replicate assays), **b** interday repeatability (5 days with three replicate assays each one) repeatability and **c** long term stability. Urease activity of 13 IU represents 100 % relative activity

the samples such as alcohol content and pH. In the first case, to rule out the effect of alcohol on the enzyme activity, the addition of up to 15 % (v/v) ethanol was evaluated. In all cases, no significant changes were found in the urease activity. Significant loss of activity was observed at pH values lower than 7 with no enzyme activity at pH 3. These results suggest that the alcohol concentration tested had no effect on the enzyme activity (both for free and immobilized urease), while neutral pH was required to obtain the highest activity level (data not shown). Moreover, the

analysis of the kinetic parameters indicates that both the apparent affinity (K_m) and maximum velocity (V_{max}) are higher in the MSNs-E system than in the free enzyme system (Table 1).

Discussion

Magnetic properties of the MSNs obtained herein are higher than previously reported ones. Indeed, MSNs prepared using Triton X-100 as surfactant, tetramethoxysilane as silica source, cyclohexane as organic solvent, *n*-hexanol as cosurfactant could be obtained in one step, but their magnetization curve reflected that the magnetization saturation was only 3 emu/g (Yang et al. 2004). Alternatively, Zhang et al. (2008) obtained silica-coated magnetic nanoparticles using Igepal CO-520 as surfactant, tetraethoxysilane as silica source, hexane as the oily phase and oleic acid stabilized magnetic particles as sedimentation nucleus. Their conclusion was that their work could not satisfy bioapplication's requirements because the magnetic silica composite had excessive amorphous silica that affected the magnetic properties of the products. The fact that MSNs-E are supraparamagnetic presents the advantage that once the magnet is removed, particles do not stay together in a clumped manner and are easily dispersed (Huang et al. 2010).

The W/O microemulsion employed in this work is thermodynamically stable and acted as reactors for hosting the polymerization reaction. Moreover, it also acted as steric barrier for inhibiting the polymerization among reactive species between different droplets during the reaction period. These effects allowed us to obtain uniform spherical nanoparticles, similar to those obtained with more complex microemulsion systems (Chang and Fogler 1997; Lopez-Quintela 2003). Indeed, MSN were previously obtained using the sol–gel process in inverse emulsions prepared using Span 80 as surfactant and tetraethoxysilane as

Table 1 Kinetic parameters of free and immobilized urease

	Free urease	Immobilized urease
V_{max} (µmol/min)	0.09 ± 0.01*	0.31 ± 0.01
K_m (µmol)	0.26 ± 0.02*	1.76 ± 0.05

*Statistical significant different from immobilized urease, $p < 0.001$

silica source. The use of Span 80 was shown to be crucial to obtain smooth monodisperse particles (Lou et al. 2008).

The enhanced activity obtained for the immobilized enzyme agrees with previous results (Ranjbakhsh et al. 2012; Robatjazi et al. 2013) and can be explained by the presence of unspecific binding sites for the substrate. In this context, the free urease reaches its maximum velocity in the presence of a high or saturated substrate concentration even though, in several cases, the substrate causes an inhibitory effect. In MSN-E, the maximum velocity is higher because the substrate can be bind to the enzyme and also to the support that favors a higher substrate concentration near the enzyme and thus promotes its catalytic activity. The adsorbent capacity of sol–gel materials is already well-known (Alvarez et al. 2011; Desimone et al. 2011) especially for compounds that form strong hydrogen bonds to silica, for example, hydrazine, urea, formamide and acetamide. This effect diminishes the overall urea concentration in the solution and thus the possibility of an inhibition exerted by the substrate is also lower. Finally, the immobilized enzyme is stable over repeated uses and storage.

Conclusion

Sub-micrometer, monodisperse magnetic silica particles were prepared using a w/o microemulsion. To the best of our knowledge, the composition of the microemulsion has not been used before to obtain enzyme-linked MSN. There are several advantages of the proposed method including the higher magnetization level of the particles obtained, uniformity of size and that they are easily functionalized to covalently-bound urease. The intra-day, inter-day and long-term stability results confirm that the procedure was successful and the MSNs-E are stable over repeated uses and storage. The maximum velocity achieved with the MSNs-E system is higher than the maximum velocity achieved with the free enzyme. These properties are a combined effect of a number of factors determined by structural changes in the enzyme as a result of its covalent binding and others resulting from the heterogeneity of the system. It is both the enzyme and the support, and the interaction between the two, that impart the system with specific physico-chemical and kinetic properties responsible for its operational performance. Solid–liquid separation of the MSNs-E can be easily achieved with a magnet allowing the reutilization of the immobilized enzyme.

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