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A Novel and Simple Alcohol-Free Sol−Gel Route for Encapsulation of Labile Proteins

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In recent years, the immobilization of biomolecules in silicate glasses formed by the sol−gel method has been attempted in a number of works,[1−3] given the good properties of the resulting matrixes; for example, preservation of the spectroscopic properties, chemical functions, ligand binding, and biocatatalysis of the encapsulated proteins, besides excellent optical properties.[4−7] However, the alcohol formed as a byproduct during the hydrolysis and condensation of the alkoxide precursors causes a detrimental effect on the activity of the entrapped biomolecules[3,8] and is an important obstacle that must be solved prior to the implementation of the sol−gel process as a universal method of protein encapsulation.

To date, just a limited number of works have attempted the design of biologically adapted sol−gel routes.[2,3,8,9] Among them, those starting from precursors such as sodium silicate[8] or glycerol-derivatized silicates[3] provide an alcohol-free sol in which the activity of the protein is preserved. Unfortunately, both routes present inherent limitations in their application; that is, the glycerol-derivatized silicate precursors need to be synthesized, while the sodium silicate precursors release high Na^+ concentration levels which must be eliminated through an acidic cation-exhange resin. Furthermore, none of these routes are suitable for the preparation of hybrid organic−inorganic matrixes that may provide friendly environments for a number of biomolecules (e.g., lipases[10] and cells[9a]).

The aqueous route proposed in this study is based on the gentle vacuum elimination of the alcohol by rotavaporization methods and can be used for the preparation of either pure silica matrixes or ormosils (organically modified silicates[11]). The use of ormosils in bioencapsulation may provide interesting properties to the host matrixes, from hydrophobic[11] to highly hydrophilic (hydrogels).[12] Rotavaporization methods have already been used in the sol−gel preparation of meso-

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porous materials.[13,14] A number of authors have reported on the convenience of the use of alcohol-free concentrated sols for bioencapsulation.[15] Surprisingly, such an experimental route has never been described to date.

The validity of the alcohol-free sol−gel route presented in this work to preserve the enzyme activity during the encapsulation process has been studied on horseradish peroxidase (HRP). HRP is a monomeric hemeprotein widely used in the field of biosensors because of its high specificity for hydrogen peroxide.[16] Its structure, function, and folding/unfolding processes are well-characterized over a wide range of solution conditions.[17,18] However, partial loss in the HRP activity (up to ≈25%) has been reported by using the regular sol−gel encapsulation processes,[19,20] as a consequence of the release of alcohol as a byproduct of the hydrolysis and condensation reactions of the sol−gel process.

The aqueous route described in this work begins with the preparation (at 4 °C) of a silica sol stock solution composed of 5.58 mL of tetraethoxysilane (TEOS, from Sigma-Aldrich), 1.9 mL of doubly distilled and deionized water, and 0.125 mL of HCl (0.62 M, from Sigma-Aldrich). The mixture was stirred vigorously for 30 min and then subjected to 20-kHz frequency and 2.4 kJ/cm^3 ultrasonic energy (Ultrasonic Processor VC100, Sonics and Materials) to obtain a homogeneous sol. The reaction conditions (sonication, $r_{w/m} = 4$ and pH = 2) have been thus selected to ensure the adequate progress of the hydrolysis reaction.[21] The removal of the alcohol (up to ≈96%, Figure 1)[22] was achieved through the application of rotavapor methods[23] on the hydrolyzed and diluted sol (1 mL of water is added to 1 mL of sol). Note that the addition of water is required to avoid the prompt gelation of the sol after removal of the alcohol. An equal volume of HRP (from Sigma-Aldrich) solution (100 mM sodium phosphate, pH = 7.4) was added to the alcohol-free sol, and the solution was placed into polystyrene cuvettes for fluorescent experiments ([HRP] = 23 μM)[24] and into the wells of a 96-well microplate (50 μL/well) for kinetic experiments ([HRP] = 11.5 nM).[25] Samples were sealed with Parafilm and stored in the dark at 4 °C for 24 h. As described elsewhere,[26] the kinetic experiments were performed in buffered phosphate solution (25 mM, pH = 6) at room temperature, keeping constant the concentration of H_2O_2 at saturation value (5 mM), while varying the concentration of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, from Sigma-Aldrich) from 0.02 to 4 mM.

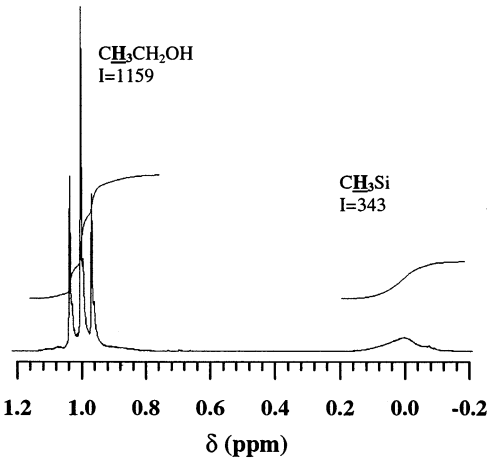

Figure 1. ^{1}H NMR spectrum of a hydrolyzed and diluted TEOS/MeTEOS sol after the application of rotavapor methods. The intensity ratio of the signal corresponding to the remaining ethanol (CH_3-CH_2OH, δ pmm 1) to the signal corresponding to the methyl groups of the MeTEOS precursor (CH_3-Si, δ pmm 0.05) is approximately 3.4. Note that the TEOS/MeTEOS molar ratio was 95/5, which corresponds to an ethoxy to methyl molar ratio of 79.

The effect of the sol−gel process on the enzyme conformation was investigated by fluorescence spectroscopy. Steady-state fluorescence has been widely used to examine the unfolding of HRP in solution[18] since it provides information about the tertiary structure of the enzyme; that is, the single tryptophan of HRP is strongly fluorescent upon denaturation, while in the native protein, the tryptophan fluorescence is quenched by the close presence of the heme prosthetic group. Steady-state fluorescence has also been used to study the denaturation of single tryptophan proteins upon encapsulation.[27,28] The spectrum of HRP encapsulated through the alcohol-free sol−gel route described in this work shows a lower intensity fluorescent band than that of HRP encapsulated through a regular sol−gel route (Figure 2), which indicates further preservation of the native structure of the protein in the alcohol-free, than in a regular, sol.[18,20]

The lack of denaturation of HRP in the alcohol-free sol−gel process must result in the complete preservation of its specific activity. The initial rates of peroxidase activity were measured as a function of the oxidized substrate (ABTS) concentration. The apparent specific activity (K_{cat} in Abs·M^{-1}·s^{-1}) was obtained through the fitting of the data to a Michaelis−Menten kinetic analysis. The K_{cat} found for HRP encapsulated through the alcohol-free sol−gel process was in the range of (or

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(22) ^{1}H NMR was performed on a Varian Gemini 200 (DMSO-d_6). The evaluation of the alcohol content was achieved after the application of rotavapor methods on a hydrolyzed and diluted TEOS/MeTEOS sol (95/5 molar ratio), using the methyl group of MeTEOS as an internal reference.
(23) Alcohol was removed from the hydrolyzed and diluted sol on a Buchi Rotavapor R200, after 25 min of controlled vacuum (20 bar) and soft thermal treatment (42 °C).
(24) Fluorescence measurements were performed at 20 °C on a 48000s SLM-Aminco spectrofluorometer (T-Optics). The excitation wavelength was 295 nm to avoid contribution of the five-tyrosine residues present on HRP. A neutral density filter was placed in the excitation path to minimize photobleaching. Fluorescence emission spectra were collected with all band-passes set to 4 nm.
(25) The peroxidase activities were monitored spectrophotometrically on a EL340 Biokinetics microplate reader (Biotek Instruments). The rate of change of absorbance at 405 nm in the UV−Vis plate reader was followed in kinetic mode, shaking the samples (final volumes = 100 μL/well) between reads.
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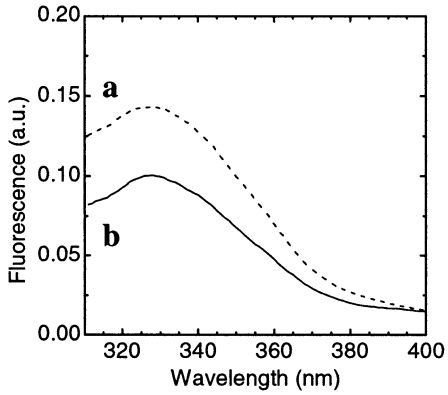

Figure 2. Fluorescent spectra of HRP encapsulated in a matrix prepared following a regular sol−gel route (a) and an alcohol free sol−gel route (b).

even slightly higher than) that found for free enzymes in solution (360 ± 20 and 310 ± 12, respectively) and notably higher than that for protein encapsulated through the regular sol−gel route (240 ± 20). Higher catalytic activities for encapsulated proteins than for free enzymes in solution have been reported as a consequence of the individual isolation of the biomolecules within the cages of the matrix.[19] The increase of the Michaelis constant value for HRP encapsulated following the alcohol-free route (2.38 mM) as compared to that for free enzymes in solution (0.55 mM) is a consequence of the diffusional resistance of the pores of the sol−gel matrixes to the transport of substrates to the enzyme.[8]

In summary, this work presents a novel and simple aqueous sol−gel route for the encapsulation of biomolecules, which are labile to the alcohol byproducts of the sol−gel process. This method is applicable to the preparation of pure silica matrixes as well as ormosils. The use of ormosils as host matrixes appears to be crucial for the development of novel materials based on bioencapsulation. The successful preservation of the native form of HRP along its encapsulation process within a pure silica matrix has been observed by fluorescence spectroscopy. Furthermore, the full preservation of the HRP activity when encapsulated through the alcohol-free route is also demonstrated by the calculation of its specific catalytic activity. A detailed study describing the suitability of this aqueous route for the encapsulation of cells in hydrophobic matrixes and hydrogels is currently in progress.

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