Recent trends in enzyme and cell immobilization by gel-entrapment and micro-encapsulation

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Introduction

Biocatalyst immobilization aims at making the best possible use of the ability of enzymes or whole cells to perform the chemical conversion of given substrates in a highly efficient manner, both technically and economically. The various methods used for biocatalysts immobilization include adsorption, covalent attachment, cross-linking and copolymerization, entrapment and micro-encapsulation. The two latter carrier-free immobilization approaches have been evaluated as alternatives in order to overcome the mass transfer limitations common to carrier-binding methods and today very popular for the confinement of biological agents, among them biocatalysts.

In recent years the continuous research efforts of finding suitable materials and strategies for efficient and cost-effective, has taken a more rational approach. The multi-disciplinary nature of immobilization has been acknowledged. As an outcome, techniques allowing for the formation of solid supports already used in other areas of knowledge, have been adapted for application in biocatalysis, as in the case of Sol-gel capsules and monoliths, and efforts are made to have information on the structure of the biocomposites. Progresses are also being made in order to improve some of classical entrapment methods. Improved biocomposites are obtained through the integration of different methods, thus overcoming limitations of single method immobilization. These developments are not only aimed at improving the catalytic ability of the biocomposites but also its non-catalytic features such as encapsulation of nano- and micro-magnetic particles that allows their easy separation from the reaction media.

With the aim to achieve those goals, recent advances in the gel-entrapment and micro-encapsulation techniques are addressed and the definition of a systematic approach as to establish guidelines that will better relate immobilization strategies and expected outcome for a given specific application. Illustrative examples regarding applications of immobilized enzymes in novel **Sol-gel** particles with magnetic properties for application in hydrolysis and synthesis of β-lactam antibiotics and fruit flavour synthesis are given: Alongside with the use of whole cells and enzymes confined in polyvinyl alcohol (**PVA**) beads, obtained through a modification of the method for Lentikats® formation, for application in several bioconversion systems, *viz.* inulin and ester hydrolysis or sterol side-chain cleavage. Another field under study in the biocatalysis research group of IBB-IST is the use of gel-entrapment methodology on development of a new protein-ionic-conducting-based materials with tailor-made properties that can open a window of opportunity for new applications in chemistry and biology, namely in electrochemical analysis. The success of this new application is based on an efficient direct electron transfer associated with tailor-made properties of these new materials combining ionic cross-link of gelatine with Ionic Liquids (IL's) and enzymes (**Ion-Jelly**). The resulting Ion-Jelly before gelification is then depositied onto electrochemical transducer or constitutes itself the transducer of amperometric biosensor.

Enzyme and cell immobilization by gel-entrapment and micro-encapsulation

1) Penicillin acylase and cutinase in Sol-gel matrix

Sol-gel is an emerging route for immobilization of biomolecules (e.g. enzymes, antibodies, cells) that involves their entrapment into inorganic silicate matrixes included in the development of biosensors and other analytical devices, stationary phases for chromatography, controlled release agents, solid-phase biocatalysts, among other applications. Additionally, Sol-gel matrixes are chemically inert, hydrophilic, and inexpensive. They exhibit higher mechanical strength, enhanced thermal stability, and negligible swelling in organic solvents, and biocompatibility and resistance to microbial attack.

A well-established sol-gel processing technique consists in hydrolyzing the adequate precursors in aqueous solutions to produce soluble hydroxylated monomers, followed by polymerization and phase separation to produce a hydrated metal or semi-metal oxide hydrogel. Removal of water from the wet gel, which is usually accompanied by changes in the structure of the pores and of the gel network, results in a porous xerogel. In this work, the preparation of Sol-gel matrix is based on hydrolysis of alkyl-alkoxysilanes and other silane derivative compounds in acid media in a sonication water bath for 10 min. This Sol-solution is then mixed with a liquid enzyme preparation containing or not nano-magnetic particles in phosphate buffer pH 7.5 obtaining the Sol-gel precursor solution. The solidification step can be carried out leaving this solution to react at room temperature and after drying and aging processes the solid Sol-gel matrix was crushed in a mortar to a fine powder. The other possibility is to add Sol-gel precursor solution into an AOT/isooctane micellar system where occur the solidification obtaining nano- and micro-particles with diameter between 25 nm and 50 μm (Fig. 1). These enzyme nano- and micro-carriers have the advantage to minimize diffusion limitation processes in biocatalysis. In both cases, the Sol-gel powder and nano- and micro-particles were stored in phosphate buffer at 4ºC before use without loss of enzyme activity during several months.

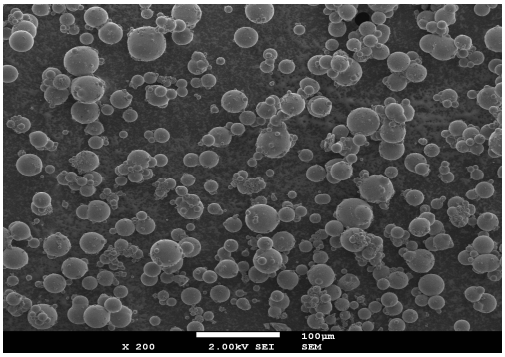


Fig. 1: SEM micrographs of the sol-gel beads with encapsulated magnetic particles and PGA.

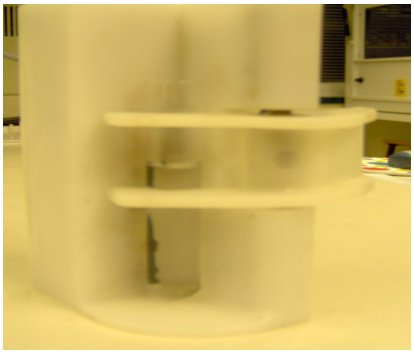


Fig. 2: Magnetic particles concentrer.

In this work particular emphasis is given to advances in penicillin acylase (PGA) and cutinase immobilization in a Sol-gel silica matrix and their successful encapsulation for the first time. A mechanically stable micro-carriers based on porous xerogels silica matrixes starting from tetrametoxisilane (TMOS) was produced. Immobilization yields of these enzymes in these Sol-gel carriers were of 95-100%, whereas, for example, the immobilized PGA activity was 60-70% at 37 ºC, as determined by the pH STAT

method. Additionally, particular emphasis is also given to enzyme immobilization by entrapment in a silica matrix with magnetic properties which enhances the recovery of the biocatalyst, in the form of nano- and micro-carriers from the reaction media (Fig. 2). Availability of inexpensive enzyme catalysts with improved specificity, activity, purity and stability is a key issue in the development of successful methodologies for the enzymatic-based synthesis of semi-synthetic β-lactam antibiotics and fruit flavours which can contribute for significant cost saving in the production of these bioproducts.

2) Several enzymes and *Rhodococcus* and *Mycobacterium* spp. cells in PVA matrix

Hydrogels are widely used for biocatalyst immobilization and their concomitant application in biotransformation systems implemented in key sectors, such as fine chemicals production, food and feed, and pharmaceutical industry. Hydrogels typically present biocompatibility, low coefficient of friction, and high water content. Although, in spite of natural hydrogels are typically biocompatible, they often display low mechanical stability and are prone to enzyme leakage. Among synthetic hydrogels, polyvinyl alcohol (PVA) is highly biocompatible and yields mechanically stable particles, produced through several methods, with different complexity. In this work a most straight forward method for the immobilization of biocatalysts in PVA particles (up to 2 mm size) (Fig. 3), based on a modification of LentiKats® liquid (GeniaLab, Germany) technology, is presented. The feasibility and prospective wide array of applications of the method is illustrated through the immobilization of enzymes (cutinase, penicillin acylase, inulinase) and whole cells (*Mycobacterium* spp. and *Rhodococcus* spp.). The resulting biocatalysts were tested in their corresponding applications, currently used as model systems at IBB/IST. The biotransformation system evaluated included inulin/sucrose hydrolysis (inulinase), penicillin G hydrolysis (penicillin acylase), and fruit flavour synthesis (cutinase), sitosterol side-chain cleavage (*Mycobacterium* spp.) and conversion of limonene-1,2-epoxide to limonene-1,2-diol (*Rhodococcus* spp.), the three later performed in the presence of organic solvents. No significant enzyme leakage was observed, even for the low molecular weight cutinase (21 kDa). Biocatalytic activity was maintained following immobilization, irrespective of the biotransformation system. Although, some diffusion limitations namely on hydrolysis of bulky inulin substrate were observed. PVA particles maintained their physical integrity even in the presence of magnetic and mechanical stirring. PVA particles could not be used at temperatures above 60ºC as melting took place. Mechanical and catalytic stability allowed for the use of the immobilized biocatalysts in several consecutive batch runs, particularly the multipurpose inulinase and penicillin acylase. The methodology is easy to implement, and relatively cheap, easily scaled and the potential for application to a wide array of biotransformation is clearly foreseen, thus presenting an alternative to currently used approaches.

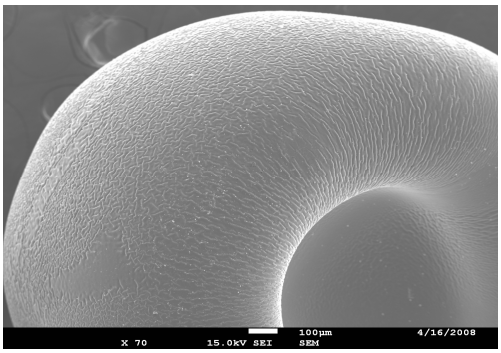


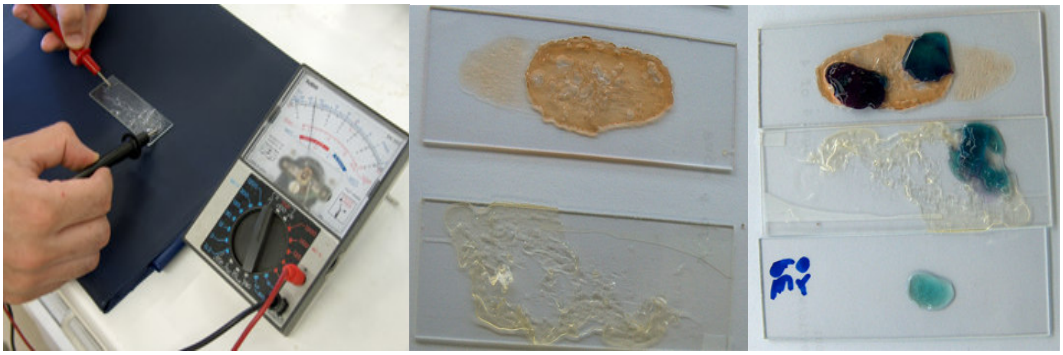
Fig. 3: SEM micrographs of a PVA-based particle with encapsulated inulinase.

3) Cytochrome, glucose oxidase and peroxidase in Ion Jelly® matrix

The development of these new materials should be performed by reaction of gelatine with an ionic cross-linker, namely Ionic Liquids (IL's) with different IL's containing cations or anions and, simultaneously, with different hydrophobicity and/or polarity.These new materials were synthesized with innovative properties, including more efficient direct electron transfer and may offer a microenvironment more compatible for biomolecules. At the same time these materials exhibit adjustable solubility and electro-conductivity when submitted to an electric potential. With this work is expected to develop models to help the design of those materials with tailor-made properties according to the composition and conditions of preparation. Additionally, we intend to investigate tailor-made properties of the new materials in terms of biocapability with a view to elucidating their success for biological application such as amperometric biosensors.

The bioelectroconductive of thin Ion-Jelly films have high amount of protein in its composition easily substituted by enzymes with great advantage to lead a much more favourable microenvironment than those obtained by carbon paste, screen-printed and electro-conductive polymers. For instance, carbon paste and screen-printed electrodes have been one of the mainstays of commercial biosensors however, some disadvantages of these materials that modified transducers are related with their low homogeneity. Furthermore, their surfaces differ widely in their ability to interface favourably with enzymes while electro-conductive polymers have been reported to have a variety of advantages in the selective deposition of enzymes but have not led to high stable amperometric biosensors.

Especial attention is focus on manufacture of these gels, especially during drying step, as it is well known that the interaction of water on the enzyme surface forms the major contribution in retention of protein structure and in turn to enzyme activity such as of peroxidase and glucose oxidase. The entrapment of both tested enzymes is in evaluation and characterization in terms of biomolecules loading and activity expressed in relation to gel composition. Electrochemistry is the basic method of evaluation of the type of enzyme interaction with the transducer surface, techniques such as linear and cyclic voltammetry are used to characterize the type of interfacial communication through the thin Ion-Jelly films produced.



a) b) c)

Fig. 4: Pictures of new materials based in ionic cross-linked (INC) a) and b) Ion-Jelly films for applications in electrochemistry and biosensors applications, c) Test of Ion-Jelly films on activity detection of glucose oxidase and peroxidase using ABTS as colorimetric dye which green and dark colour reveal visually a strong enzyme activity.