Bioencapsulation Research Group

Bioencapsulation Innovations

December 2013

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EDITORIAL

Enzyme encapsulation

At the middle of the 90s, Luis P. Fonseca started to work intensively on the production and purification of enzymes a latter on biocatalysis using encapsulation as a straightforward and reproducible method on enzyme immobilization.

Enzymes have been used throughout human history and today the enzyme applications have considerable role in the heart of biotechnology processes.

A large number of these biotechnology processes require a successful enzyme immobilization in terms of resistance to leaking, retention of enzyme activity as long-term storage and operational stability under adverse environmental conditions, accessibility to substrates, fast catalysis, and, in general, high enzyme immobilization density and adequate orientation.

Among the different methods of immobilization, enzyme encapsulation inside of a host semi-permeable membrane or entrapment in a network matrix such as hydrogels and other polymeric materials in form of particles, capsules, fibers, etc, is of particular interest. Additionally, the enzyme encapsulation processes are straightforward and reproducible and does not require sophisticated equipment.

These enzyme encapsulation methods use very mild conditions that hardly affect enzyme intrinsic biocatalytic activity and allow its confinement without totally loss of its freedom but restrict unfolding movements. Additionally, enzyme encapsulation somehow mimics their natural mode of occurrence inside of the cells and provides a protective environment to the changes in the operating parameters. Other advantages of encapsulation are the permeability of the matrices, which allows the transport of low-molecular weight compounds without leaking of the entrapped enzymes, the tuneable material porosity which allows accommodation of enzymes of different size, the possibility of chemically modifying the matrix for tailor-made microenvironment more adequate for specific biocatalysis or controlled enzyme release in case used as drug-delivery systems, or design new matrices with smart properties or more resistance to chemical, thermal and biological degradation, and the negligible swelling effects.

According these advantages, enzyme encapsulation finds ever-increasing application in a wide variety of fields such as medicine and controlled release delivery systems, biosensing and clinic diagnostic, biocatalysis in the manufacture of high-value products including pharmaceuticals, flavors and fragrances, specialty and fine chemicals, and other low- and middle-value products on agriculture, food, detergence, beer and beverage industries, biofuels, among many others.

Recent interest in nanotechnology has also provided a wealth of diverse nanoparticles and nanoscaffolds that could potentially support enzyme encapsulation and immobilization. These nanoscale structures reduce diffusion limitations and maximize the functional surface area which allows increasing the enzyme loading. One possibility is to use it to encapsulate the enzyme into micro- and/or nano-meter sized biocatalysts reactors.

Recently, one of these works has led to development of micro- and nanospheres of sol-gel using an emul-

EDITORIAL (CON'T)

sion technique and simultaneously encapsulating of penicillin acylase and magnetic nano-powder. Penicillin acylase was successfully used on the synthesis of cephalexin in aqueous media while the magnetic properties of the sol-gel micro- and nano-spheres allow easily biocatalyst separation through a magnetic concenter.

Today, the most challenging aspects of enzyme encapsulation continues to be on the development of the matrix materials and encapsulation methods for enzyme integration in the host matrix and retaining full activity and increase stability. Unfortunately, the structural basis of enzyme encapsulation in molecular compartments of matrixes starts only to be understood and, for this reason, the drawback of enzyme encapsulation is there demanding a long-time trial-and-error process to optimize it for specific application.

In any case, the coming years seem to be very important and exciting to use methods and new and smart materials that easily anticipated encapsulated enzyme behavior and contribute to propose and emerge as standards for most significant bioencapsulation applications.

We hope that in the current issue will get you informed in this field of enzyme encapsulation and interested in some of exciting examples.

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PROFESIONNAL NEWS

Senior appointments at Appvion

Appvion Inc, formerly Appleton Papers Inc, has appointed Matt Denton as senior vice president and general manager of the company's carbonless and security papers business and Jason Schulist has joined the company as vice president of continuous improvement.

More information: http://prn.to/1cKRWdd

Continued market growth for encapsulation in the food and beverage industry is forecast

A new market survey from "Markets and Markets" Global Food Encapsulation Market (2012 – 2017) - By Types, Functions, Applications, Ingredients, Shell Materials, Packaging, & Geography: Trends & Forecasts, predicts a growth that will mean the global market could be worth 42 billion US dollars by 2016. Details can be found at http://bit.ly/1dfWXsc. A report on the wider microencapsulation industry is also due to be published "Microencapsulation Market - Global Industry Analysis, Size, Share, Growth, Trends And Forecast, 2013 – 2019" by Transparency Market Research.

More information: http://bit.ly/IYk9BQ

Microencapsulation start-up awarded £1 Million in seed funding

Aqdot Ltd, a spin out of Cambridge University, UK has successfully secured funding for its next stage of development only one year after being formed. The company claims its new platform technology will provide a range of benefits over existing technologies including a quick "one-step" process that will provide scope for cost savings, and options for tailoring triggered release. More details can be found at http://bit.ly/1b8J3W7 and at the company's website www.aqdot.com

More information: http://bit.ly/1b8J3W7 , www.aqdot.com

Erytech Pharma valid its therapy against blood cancers

Erytech Pharma has obtained the authorization to test its drug encapsulation in blood red cells for testing treatment of a leukemia form which concerns more than 34 000 persons per year in USA. This innovative approach allows to reduce secondary negative effects and the treatment of fragile patients

More information: http://bit.ly/1fUje1i

CALENDAR

How To Make Nanocapsules
February 5-7, 2014
Henderson, NV, USA
http://bit.ly/19lGaA4

Fluid bed processing
March 11-13, 2014
Binzen, Germany
http://bit.ly/1fITmZg

9th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology
March 31 - April 3, 2014
Lisbon, Portugal
http://worldmeeting.org

Granulation & tabletting
April 8-10, 2014
Binzen, Germany
http://bit.ly/1fITmZg

SCT-SFNano Joint Meeting
April 8, 2014
Paris, France
http://bit.ly/1cjBQmy

Specialized Training Course for Encapsulation of Animal Cells
April 24-25, 2014
Zurich, Switzerland
whelehan.m@buchi.com

Fonctional film coating
June 3-5, 2014
Binzen, Germany
http://bit.ly/1fITmZg

ProtStab2014 10th International Conference on Protein Stabilisation
May 7-9, 2014
Stresa, Lake Maggiore, Italy
http://www.protstab2014.it/

DynaCaps2014
July 15-18 , 2014
Compiègne, France
http://www.utc.fr/dynacaps2014/

20th Intenational Symposium on Microencapsulation
October 3-4, 2015
Boston, USA
Web site available Soon

6th Training School on Bioencapsulation

March 4-7, 2014 - Nha Trang, Vietnam
http://bioencapsulation.net/2014_Nha_Trang/

17th Microencapsulation Industrial Convention

April 23-25, 2014 - Bruxelles, Belgium
http://bioencapsulation.net/2014_Brussels

22th International Conference on Bioencapsulation

September 17-19, 2014 - Bratislava, Slovakia
http://bioencapsulation.net/2014_Bratislava/

2nd South American Workshop on Microencapsulation

November 2014 - Joa Pessao, Brazil
Web site available soon

ARTICLE

SPINCHEM: A NOVEL REACTOR CONCEPT FOR BIOCATALYSIS

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INTRODUCTION

For biocatalysis to become an emerging field in organic synthesis, immobilization of catalysts becomes more and more important (Bornscheuer, 2012). This is especially the case in large-scale processes where enzyme immobilization or encapsulation of whole cells is still the most commonly used technique to improve long-term stability and recyclability, however protein engineering solutions can also be applied to enhance these features. For standard reactor setups a simple stirred tank reactor (STR) or a fixed bed reactor (FBR) are usually employed. Although the STR is probably the simplest reactor, their main problems are the mechanical forces that occur during stirring, which can destroy the catalyst during longer reaction times. Furthermore, for product isolation, separation of the solids from the reaction medium is necessary. In the laboratory this is carried out by filtration or centrifugation, but in industry such process steps should preferably be avoided. In the FBR the catalyst is packed as a fluidized bed. However, process control and reactor setup become more challenging due to pressure drops, pH gradients or catalyst saturation by reaction components. The aeration of the reaction medium is also rather challenging in a FBR. To overcome these problems the rotating flow cell, SpinChem (SCR, model S6530, SpinChem is a registered trademark of Nordic Chemquest AB) was developed.

The SpinChem concept

An outstanding advantage of the SpinChem over conventional reactor setups is the protection of the (bio)catalysts, while simultaneously ensuring efficient mixing of the reaction medium. For this, the catalysts are packed into a specially designed compartment, which is mounted to an overhead-stirrer (Fig. 1). By stirring, all reactants are passed through the catalyst bed due to centrifugal forces. This way the capsules or particles are completely protected from any mechanical forces, which occur from stirring. Furthermore, downstream processing becomes much simpler; no separation of the catalyst from the reaction medium by filtration or centrifugation is required.

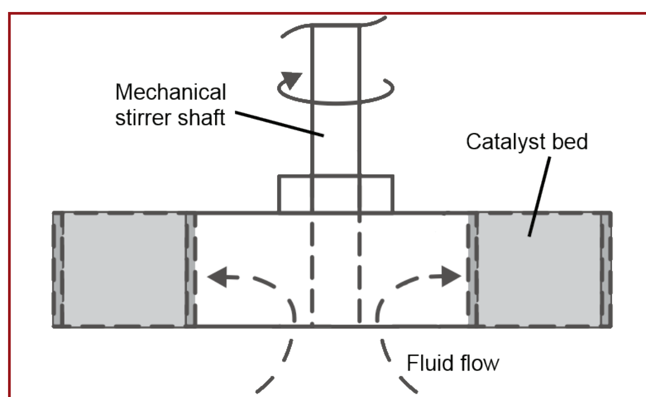

Figure 1. Schematic drawing of the SpinChem reactor.

Biocatalytic reactions

To investigate the performance of the SpinChem reactor concept, we compared three different reaction types, of which each was carried out using the SCR, as well as a conventional STR and a FBR, in which special focus was given to reusability of the biocatalyst and its stability. All experimental details can be found in the recent publications (Mallin, 2013b-c). We investigated the kinetic resolution of a racemic amine using a (*R*)-amine transaminase (*R*-ATA) covalently immobilized on chitosan, a transesterification with an adsorptive immobilized lipase in an organic solvent and the oxidation of a ketone using encapsulated whole cells harboring a Baeyer-Villiger monooxygenase (BVMO) to yield ε-caprolactone (Figure 2).

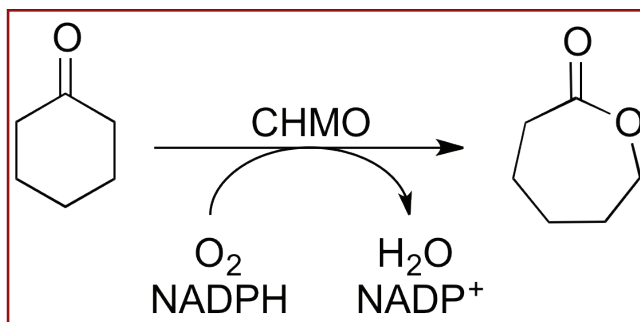

Figure 2. Oxidation of cyclohexanone to ε-caprolactone using encapsulated whole cells harboring a Baeyer-Villiger monooxygenase (CHMO).

RESULTS

Encapsulation of *Escherichia coli* BL21 in calcium alginate

The immobilization of isolated BVMOs often revealed many challenges as the immobilized enzymes often had specific activities which were too low, and the need for a cofactor recycling system made their application more complicated. In a recent review (Balke, 2012) these problems were discussed in more detail. The same experiments with isolated BVMOs (e.g. a CHMO from *Acinetobacter calcoaceticus*) immobilized on several carriers using different methods were performed. It was concluded that an encapsulation of whole cells bearing the CHMO for the desired reaction would be the best solution. Prior to encapsulation, the expression

of the CHMO in the host cells was optimized by performing experiments at various temperatures and for different time periods. After induction at an optical density of 0.8 to 1, expression was performed at 30°C for 5 to 6 hours. After harvest, the free cells were permeabilized using different reagents (CHAPS, CTAB, SDS, Triton™ X-100, DMSO, Tween® 20, PEI). Here, 1 % DMSO for 30 min at 4°C resulted in 40 % more activity (free cells: 5.4±0.4 U/g). Following a protocol from Zhang et al. (Zhang, 2010) for the encapsulation, a cell mass of 50 g/l and a calcium-alginate concentration of 1.8 % were used. This encapsulation process reduced the activity to 28 % compared to the free cells, which was mostly attributed to diffusion limitations. Next, the different reactor types were studied for the BVMO-catalyzed biotransformation.

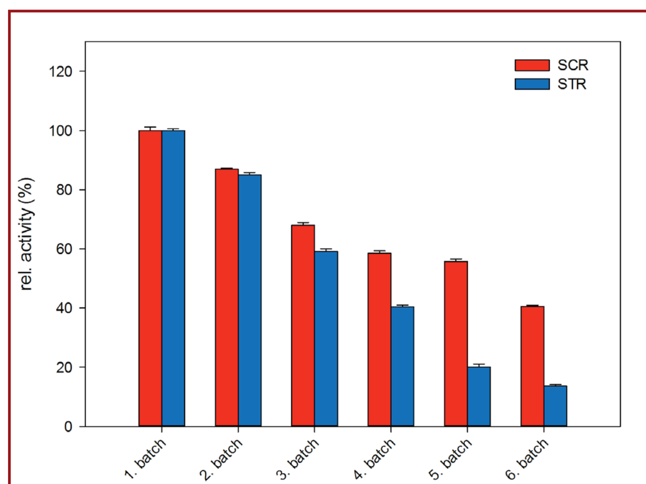

Figure 3. Comparison of the SCR with the STR in recycling studies for the CHMO-catalyzed biotransformation using encapsulated *E. coli* whole cells.

Biocatalysis with the SpinChem reactor

Using the FBR we experienced low conversion using immobilized *R*-ATA or encapsulated CHMO bearing cells; in the latter case with a 9-fold lower conversion. Although we found similar conversions comparing STR and SCR for all three reactions, long-term reactions with several consecutive batches clearly showed advantages of the SpinChem reactor compared to the stirred tank reactor. In the *R*-ATA reaction the SCR resulted in 31 % higher residual activity of the enzyme compared to the STR after five batches (Mallin, 2013b). For the encapsulated whole cells a residual activity of 41 % was found for the SCR compared to only 14 % for the STR after six batch reactions (Figure 3). Here, the addition of 10 mM $CaCl_2$ to the reaction solution was found useful to enhance the stability of the capsules. Furthermore, downstream processing was much easier with the SCR as only a simple washing steps is required compared to the classical stirred tank reactor, where filtration or centrifugation are needed to separate the biocatalyst.

DISCUSSION & OUTLOOK

We investigated the application of a new reactor concept for biocatalysis (with transaminase or lipase) and biotransformation (for encapsulated *E. coli* whole cells containing a BVMO). Three different reactions and immobilized types of biocatalysts were compared. The SpinChem reactor showed equal or superior performance especially in consecutive batch experiments, and the enhanced mechanical stability of the immobilized enzyme compared to the STR could be demonstrated. Additionally, the downstream and washing process was much more simplified.

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Hendrik Mallin received his diploma degree in Biochemistry from the University of Greifswald in 2010. Currently, he is PhD student (financed within the Biokatalyse2021 cluster, FKZ:0315175A) at the Institute of Biochemistry in the group of Prof. Uwe Bornscheuer. His research interests are biocatalysis, immobilization and protein engineering of biocatalysts involving mainly transaminases and oxidative enzymes. Jan Muschiol also received his diploma degree in Biochemistry in Greifswald in 2012. Currently, he is PhD student at the same group financed by the DFG (Bo1862/8-1). His research interests are enzymatic reaction cascades involving monooxygenases and their protein/process engineering.

ARTICLE

ENZYME ENCAPSULATION IN BIOACTIVE PAPERS

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INTRODUCTION

Bioactive papers are obtained through the modification of cellulosic substrates with various types of biomolecules. When properly designed, these functionalized papers can be used as sensors to detect the presence of toxic compounds, as bioactive filters to capture and degrade water pollutants, or even as antimicrobial towels to promote better sanitization and hygiene. Recently several examples of papers modified with printed organic conducting polymers have appeared in the literature, and have shown the possibilities to develop paper-based electrochemical biosensors and biofuel-cells. The motive behind using paper as a substrate to develop such new devices, lies in the wide availability of paper, its low cost, its ability to soak and channel water samples by capillarity, and its manufacture in large amounts, with the production process been adaptably to incorporate the appropriate biomolecules. In addition the added value of bioactive papers, in comparison to unmodified papers, is very appealing to paper manufactures as it gives them a higher-value product, which has a significant commercial advantage over competitor products.

The modification of paper with biomolecules, especially at larger industrially-relevant scales, presents several technological difficulties that must be addressed before bioactive papers can become a reality, and enter the market as common products. These difficulties are related to the very nature of biomolecules. Enzymes, antibodies, and other proteins were designed to strive in cells and organisms rather than on the surface of paper, which is exposed to many different environmental conditions, resulting in their activity decreasing. Common conditions for the industrial paper-making processes include high temperatures and high shear rate steps.

Our strategy to address these issues is to encapsulate the biomolecules, allowing their protection against degradation during the immobilization procedures like coating, and to maintain their activity over extended storage periods. The aim of this communication is to present an overview of the encapsulation procedure we used to modify paper substrates on a small scale, and to demonstrate how this technique can be scaled-up to larger substrates, using conditions that mimic those used in the industry. The enzymes selected for the development of the immobilization platform are laccase and glucose oxidase, and particular attention is paid to the thermal stability of these enzymes in poly(ethyleneimine) microcapsules.

ENZYME ENCAPSULATION

Microcapsules were prepared by interfacial reticulation of polyethyleneimine using sebacoyl chloride as the crosslinking reagent, using either a water/cyclohexane emulsion-based method or a technique that employs a commercial vibrating nozzle type encapsulator. (Zhang, 2010). Figure 1 shows a comparison of the typical microcapsules obtained by both methods. The images in green were obtained by confocal laser scanning microscopy (CLSM) with poly(ethyleneimine), PEI, modified with fluorescein isothiocyanate (FITC). A general observation from the results in Figure 1, is that the vibrating nozzle encapsulator (equipped with a 100 µm nozzle) will produce larger capsules (200 µm diameter) with a small size distribution, while the emulsion-based technique will allow the production of capsules with a smaller average diameter (20 µm) but at the expense of a larger size distribution.

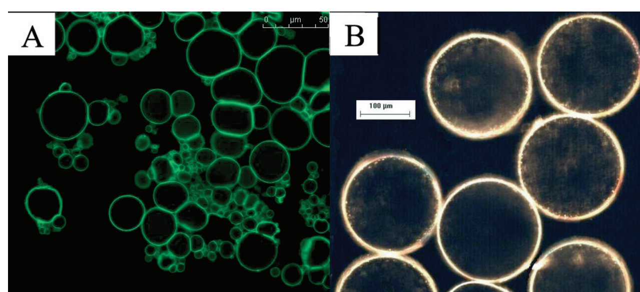

Figure 1. Comparison of the PEI microcapsules obtained by the emulsion (A) and encapsulator (B) techniques.

Both techniques were found to decrease the specific activity of the enzymes laccase and glucose oxidase. In the case of laccase, fluorescence and circular dichroism experiments revealed that PEI affected the protein structure, most likely due to a complexation of the polymer with the copper atoms found in its active sites (Zhang, 2011). During the encapsulation procedures, proteins were found to interact strongly with the polymer due to ionic attractive interactions. Proteins have a globally negative charge and PEI is positively charged at the pH used for encapsulation. These interactions caused the proteins to be found mainly within the membrane of the capsules, as evidenced in Figure 2 (Kouisni, 2009).

The PEI-laccase complexation, combined with a slower substrate transport to the encapsulated enzyme across the membrane of the microcapsules, resulted in a 65% decrease in laccase specific activity vs. free enzyme in solution. This deleterious effect of the polymer was not observed with glucose oxidase, which does not contain metal atoms and the decrease in activity was only attributed to the substrate diffusion restriction.

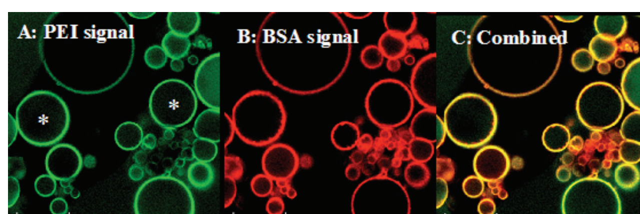

Figure 2. CLSM images of proteins located within the wall of microcapsules. The green emission is from PEI (FITC-tag) and the red signal is for proteins (BSA tagged with Texas red).

LAB-SCALE MODIFICATION OF PAPER

Comparing both encapsulation techniques, it appeared that the emulsion method was the best suited to produced the enzyme containing microcapsules for immobilization on paper. This technique produced capsule with smaller diameters, which enabled a better insertion and retention of microcapsules on the surface and within the paper sheet.

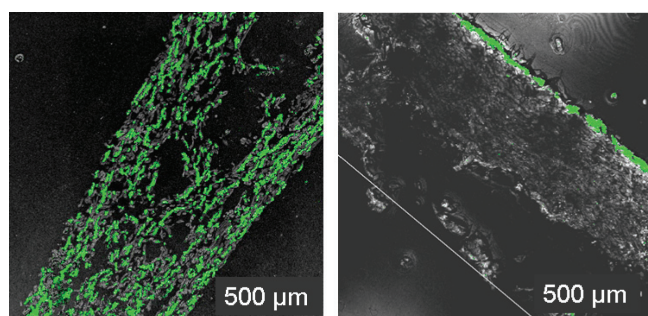

Figure 3. Fluorescence microscopic images of thin sections of papers modified with FITC-labelled PEI.

The successful incorporation of fluorescently-labelled microcapsules in the bulk and on the surface of a paper substrate is showed in Figure 3. These microcapsules were also found to be highly efficient in maintaining the enzymes in the paper sheets. When laccase was simply adsorbed on the surface of paper, washing the sheet with 200 mL/cm^2 removed 94% of the enzymes. However, if laccase was encapsulated and the capsules used to modify the paper sheet, the same treatment resulted in a decrease of only 5%.

Figure 4 shows an example of the colorimetric signal that can be generated from papers modified with laccase microcapsules. In these samples, various concentrations of *p*-phenylenediamine were added and the images were taken at different time intervals. A colour visible by eye was noticed within the first few minutes.

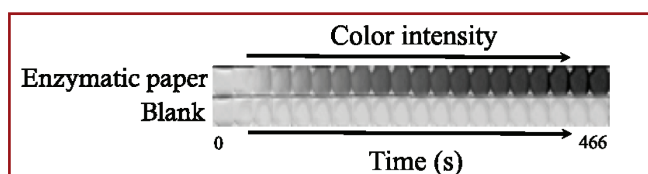

Figure 4. Oxidation of p-phenylenediamine by a paper modified with encapsulated laccase.

LARGE-SCALE MODIFICATION OF PAPER

The paper samples described above were made in the lab by firstly adding the capsules in a suspension of bleached paper pulp and by collecting the pulp on a filter. This technique is efficient for fast lab-scale evaluation of immobilized enzyme activity but would not be applicable on a large scale paper-making machine, due to the losses in microcapsules and the large volumes of water required. In order to evaluate the applicability of microcapsules to modify large surfaces of paper and to maintain the enzymatic activity during industrially-relevant processes, we used a cylindrical laboratory coating machine (CLC) to coat a starch-based suspension of microcapsules on 0.75 x 3 m paper sheets (Guerrero, 2011). In these larger scale experiments, a batch of 1 kg of laccase microcapsules was successfully prepared and applied to the paper sheets. The CLC uses a blade to disperse the coating suspension on a rolled paper sheet, providing speeds (500-800 m/min) and shear rates comparable to those found in industrial coaters. The coated paper sheet is dried under an IR lamp, which is also representative of industrial conditions. As the shear and drying can significantly affect the structure of proteins, this procedure will allow a more realistic assessment of the enzyme activity. Four papers were coated with the CLC and allowed to dry under the IR lamp at 100% of its power (36 kW) for various periods of time, from 0 to 30 s. The colorimetric analysis of the response of each sample shows very little differences in laccase activity between 0 and 20 s and a 20% decrease for 30 s of IR exposure.

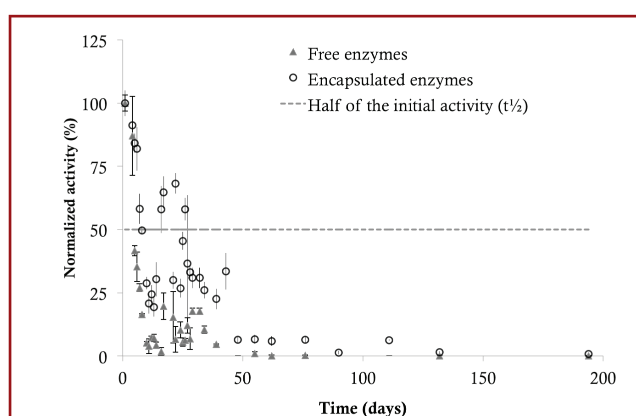

Figure 5. Storage time of a laccase-modified paper is enhanced using microencapsulation.

The activity of coated paper was sampled over a period of 6 months to evaluate if microencapsulation provides better activity retention upon storage at room temperature. Figure 5 illustrates the normalized residual activity of laccase coated with the CLC, over a 6-month period. While both samples completely lost their activity after 132 days, the microencapsulation provided a considerably better stability of laccase during the first months of storage at room temperature. Microencapsulated laccase reached 50% of its initial activity after 28 days, in contrast to only 4 days with the paper coated with free laccase. This beneficial effect of microcapsulation is due to the restricted exposure of the encapsulated enzymes to protein-degrading microorganisms or biomolecules.

CONCLUSION

An efficient enzyme immobilization is crucial in the development of bioactive papers to maintain the enzymatic activity during the paper-making process, as well as for long storage times. As we demonstrated in this short communication, microencapsulation is a well suited technique to accomplish these tasks. Since numerous microencapsulated products are already manufactured industrially and commercialized, its employment to modify large amounts of papers with enzymes is realistic, and will play a role in the future for the development of bioactive paper sensors or filters.

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Dominic Rochefort carried out his Ph.D. research (2002, U. Montréal) on the electrocatalysis of laccase. After a postdoctoral fellowship (INRS and Cornell) on materials for energy storage and conversion, he went back to U. Montréal as a Professor in 2004. His research involves ionic liquids as electrolytes for energy storage and microencapsulation of biomolecules in bioactive papers.

THESIS ABSTRACT

Formation of Alginate Nanospheres

Jerome P. Paques

Affiliation Wageningen University

Date/place 15/01/2014 - Wageningen, Netherlands

Supervisor Cees J. M. van Rijn, Erik van der Linden, Leonard M. C. Sagis

The formation of small alginate particles with a diameter well below 25 µm is described. Using two different methods we formed gelled alginate spheres around 1 µm. Also nanospheres as small as 200 nm and even smaller have been realized. The particles can be used for encapsulation purposes in food products. The pH conditions inside these particles are sufficiently mild such that pH sensitive components, such as peptides, probiotics, etc. can be encapsulated. The alginate micro- and nanospheres are formed by emulsifying an alginate solution in an oil phase followed by both internal and external gelation of the spheres. A novel external gelation method is developed where $CaCl_2$ nanoparticles are dispersed in the continuous oil phase. Energy-dispersive X-ray spectroscopy and Auger electron spectroscopy show that these nanoparticles migrate to the emulsion droplet interface, where they dissolve into the aqueous alginate phase and cause gelation, forming gelled spheres. Gelation of the spheres is confirmed with a novel technique using Congo red as an indicator. In another method $CaCO_3$ nanoparticles together with glucono delta-lactone (GDL) are used for internal gelation of the alginate spheres. The pH evolution as a function of time and rheology are determined during gelation of macroscopic alginate gels. $CaCO_3$/alginate mass ratio and GDL/$CaCO_3$ molar ratio are optimized. Furthermore SEM analyses is performed for characterization of the alginate spheres.

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Microencapsulation of probiotic bacteria : Mechanisms of formation by a multi-scale approach

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Date/place 10/12/2013 –Nancy, France

Supervisor Pr Joël Sscher, Dr Claire Gaiani

An encapsulation process for probiotic bacteria using only milk proteins is developed. The laboratory scale followed by a pilot and an industrial scale development of the process allow the production of stable and resistant microparticles both in aqueous and gastric media. The nature and quantities of proteins added is found to influence the bacterial location in the microparticles. A molecular study of interactions between milk proteins and bacteria by atomic force microscopy reveals that whey proteins can specifically interact with bacteria in contrast to caseins. The presence of piliated bacteria is found favorable to establish strong and long interactions with proteins. This work permits the interpretation of results obtained at a macroscale thanks to microscopic observations and nanoscopic interactions study. This multi-scale approach permits the elucidation of mechanisms driving the probiotic encapsulation in milk matrices

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ARTICLE

SYNTHETIC GELS FOR ENZYME ENCAPSULATION: PVA AND SOL-GEL MATRICES

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ENZYME ENCAPSULATION IN APPLIED BIOCATALYSIS

Immobilization of enzymes requires the selection of a suitable approach. Factors that are essential in the selection are: a) retention of catalytic activity; b) resistance to enzyme leakage, while allowing easy access to substrate and c) long-term storage and operational stability. Encapsulation is one of the methods for enzyme immobilization that has gathered particular attention, since usually: a) it involves simple and straightforward methods; b) does not require sophisticated equipment; c) is fairly reproducible and d) allows tuning of morphological, optical and chemical properties of the matrix. Two commonly used approaches for enzyme encapsulation involve the use of either hydrogels or sol-gels. These approaches will be discussed in the following section in view of industrial applications.

POLYVINYL ALCOHOL: A SYNTHETIC HYDROGEL FOR ENZYME IMMOBILIZATION

Hydrogels from either natural or synthetic macromolecular polymers have been widely used for enzyme immobilization. Since synthetic hydrogel carriers typically display higher chemical stability and are less prone to abrasion than natural hydrogel carriers, their use may prove advantageous under operational conditions. Polyvinyl alcohol (PVA) is a cheap, easily available and biocompatible synthetic polymer, which has been gaining relevance for application as a biomaterial. The formation of PVA hydrogels for enzyme immobilization can be achieved through: a) UV irradiation; b) freeze-thawing cycles; chemical cross-linking; controlled dehydration, so that hydrogen bonding is promoted. The later approach was disseminated by a particular formulation of PVA and polyethylene glycol (PEG) labeled as LentiKats® commercialized by GeniaLab. Later, large scale production and applications involving this particulate material has made been available by Lentikat´s (http://lentikats.eu/en/).

While the canonical approach for the production of these lens shaped particles involves drying under controlled temperature and humidity, a different approach was undertaken at our research group where LentiKats liquid, once mixed with the enzyme solution, is extruded into PEG (Fernandes et al. 2009).

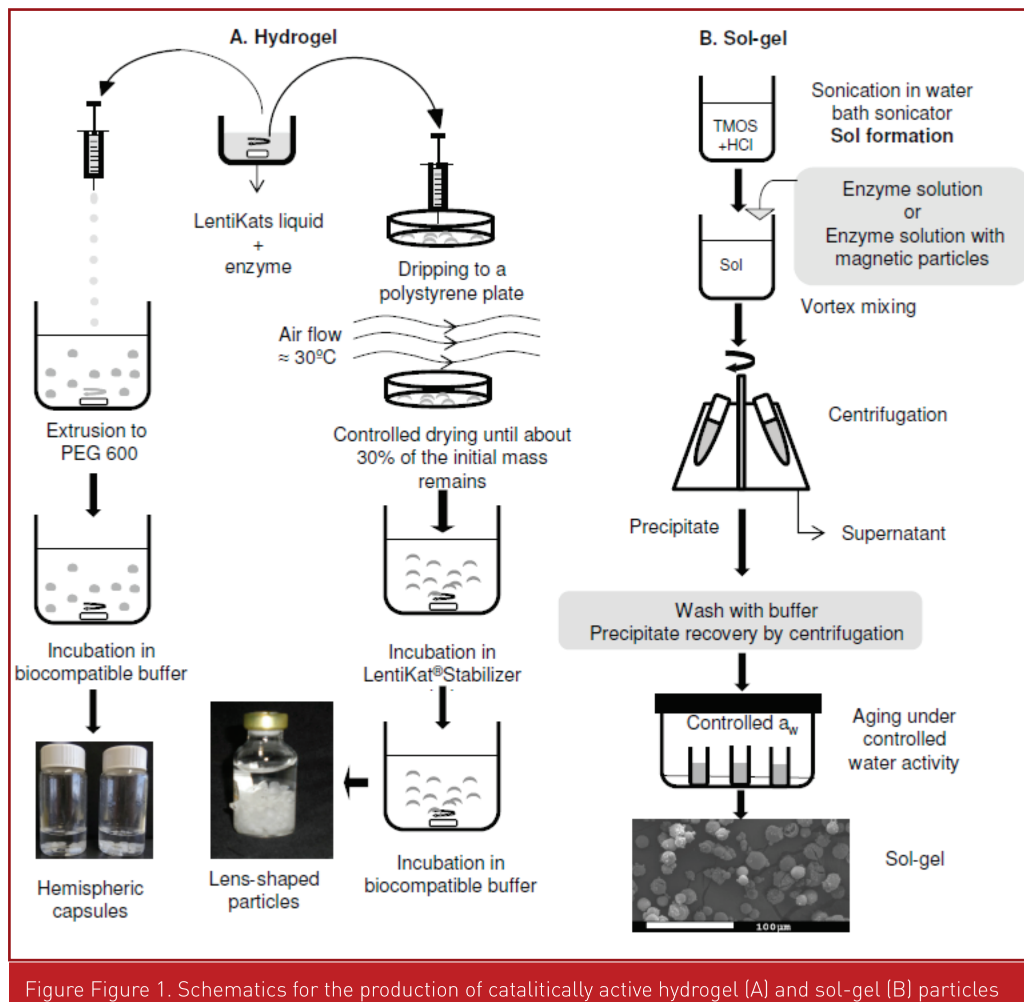

Figure Figure 1. Schematics for the production of catalitically active hydrogel (A) and sol-gel (B) particles

The resulting capsules (PVA-PEG) are hemispheric shaped, rather than lens-shaped (Figure 1 A). As happens with the standard dehydration method, the modified method implemented is quite straightforward, easily reproducible and can be easily scaled-up, namely in manners reminiscent of the classical procedure for gelation of calcium alginate in bead form. Moreover, PEG can be recycled with no noticeable negative impact in encapsulation efficiency.

Both hemispheric shaped capsules, as well as standard lens-shaped particles have been tested with success for the immobilization of several enzymes, the resulting biocatalysts being the core of bioconversion processes of relevance wit-

ARTICLE

Bioconversion system/enzyme	Immobilization method	Comments	Reference
Sucrose hydrolysis/invertase	PVA/PEG	High operational stability: 90% of product yield after 20 consecutive 24h-batch runs at 50ºC of 100 g/L sucrose solutions. Initial product yield of 100%. Mild (1.8 fold) increase in KM after immobilization	Fernandes et al, 2009
Inulin hydrolysis/inulinase	PVA/PEG	High operational stability: 70% of product yield after 20 consecutive 24h-batch runs at 50ºC of 50 g/L inulin solutions. Initial product yield of 90%. Mild (1.9 fold) increase in KM after immobilization.	Cattorini et al. 2009
Inulin hydrolysis/inulinase	PVA/Lentikats	Good operational stability: 68% of product yield after 10 consecutive 24h-batch runs at 50ºC of 50 g/L inulin solutions. Initial product yield of 90%. Mild (1.6 fold) increase in KM after immobilization.	Cattorini et al. 2009
GOS synthesis/ β-Galactosidase	PVA/Lentikats	Continuous operation in in a packed-bed reactor (PBR) reactor at 40ºC. GOS production corresponded to 300g/L of total sugars with a feed rate of 10.8 mL/h lactose solution (400 g/L). GOS productivity of 117 g/Lh.	Jovanovic-Malinovska et al. 2012
H2O2 reduction/ Catalase	PVA/PEG	Integration in a continuous, bi-enzymatic process for oxidation of cholesterol to cholestenone. Immobilized catalase particles were piled in a PBR and reduced the toxic H_2O_2 formed, the set-up allowing for 300 hours of operation time, corresponding to an end production of 36 M of cholestenone	Marques et al. 2012
PG hydrolysis and cephaxelin synthesis/PG acylase	TMOS xerogel	High operational stability: 100% activity retention after 10 consecutive batch runs for PG hydrolysis. The activity remained roughly unaltered for 6 months under storage at 2-8 ºC. Tetramethoxysilane (TMOS) proved a better precursor than methyltrimethoxysilane (MTrMOS) or of a mixture of both. Physical degradation of xerogel micro-particles was negligible for operation under mechanical agitation or in a PBR. The immobilized biocatalyst had a projected half-life of 133 h when used for cephalexin synthesis 14 ºC.A maximum specific productivity of 5.9 mmol/ h $g_{biocatalyst}$ was obtained.	Bernardino et al. 2011

Table 1. Some representative bioconversion systems successfully developed at the Department of Bioengineering, IST, using PVA and sol-gel based encapsulated biocatalysts.

hin the scope of: a) food and feed, viz. sucrose and inulin hydrolysis, synthesis of galacto-oligosaccharides (GOS); b) pharmaceutical sector, viz. production of intermediate steroids and c) renewable resources, cellobiose hydrolysis. Further details on selected systems are given inTable 1. In either method, there was no need for any kind of additional processing prior (viz. formation of cross-linked enzyme aggregates) or after immobilization, to minimize enzyme leakage. Moreover, no chemical agents, that could prove deleterious for enzyme activity/stability, are required for gel formation.

In most cases PVA biocatalysts were used in singe-enzyme systems but recently PVA-entrapped catalase was used cooperatively with cholesterol oxidase so that a continuous, miniaturized system for cholestenone production from cholesterol was implemented (Fig. 2, Table 1). Given the lipophilic nature of steroid like molecules, sterol oxidation was carried out in an organic-aqueous two-liquid phase system, flown through a Y-shaped microchannel reactor, using cholesterol oxidase (COx) as biocatalys. Toxic hydrogen peroxide, formed as by-product partitions into the aqueous phase, where it flows alongside with COx, until hydrogen peroxide is reduced by the immobilized catalase, packed in a fixed bed reactor, thus allowing for COx to be recycled to the microchannel reactor.

In all cases, PVA based biocatalysts have exhibited high operational stability, provided that temperatures of incubation which did not exceed 50ºC, and encapsulation had minor impact in the intrinsic catalytic features of the immobilized enzymes, the whole suggesting a high potential for application in large-scale processes.

SOL-GEL MATERIALS AS ENZYME CARRIERS

Enzyme encapsulation was almost exclusively performed using hydrogels as carriers, until suitable methodologies were developed that allowed the formation of inorganic glass materials doped with biomolecules at room temperature. These sol-gel materials, when doped with enzymes are of particular interest for applied biocatalysis: the glass is chemically, thermally and photochemically stable, allowing for operation in a wide diversity of environments; it is transparent into UV range, which can be advantageously used for integration with analytical methods; key properties, viz. hydrophobic/hydrophilic nature and porosity, can be suitably tuned, namely given the right selection of the precursors. In our research group silicilic acid-like derivatives have been favored as precursors. Early research efforts focused on the development of competitive biocatalysts to be used in the production of semi-synthetic antibiotics. Thus, a straightforward, scalable and easily reproducible methodology was developed for the production of micro particle

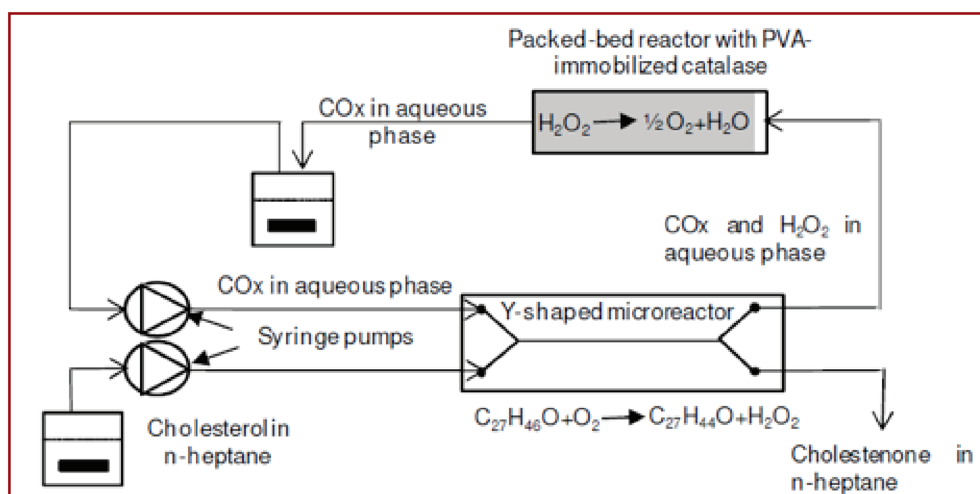

Figure 2. Schematics for the cholesterol oxidation to cholestenone promoted by COx, coupled to the reduction of hydrogen peroxide, promoted by PVA/PEG entrapped catalase.

xerogel biocatalyst (Figure 1 B), using penicillin G acylase (PGA) as enzyme model and penicillin G (PG) hydrolysis and cephalexin synthesis as reaction systems (Table 1). Throughout the process, the relevance of several parameters in the production of resilient and catalytically active biocatalysts were established, namely: the operational conditions for aging, viz. time, temperature and water activity environment; the effect of the nature of precursors on the hydrophilic/hydrophobic nature and porosity of the xerogel; the pH environment and mechanical stress on the durability of the xerogel micro-particles. Ultimately, a xerogel based in tetramethoxysilane (TMOS), produced by a microemulsion method, with a ratio of the enzyme solution to precursor solution of 1.6:1 (v/v), aged for 7 days at room temperature, under a water activity environment of 0.75, proved to be the most effective, provided operation was performed in an environment under pH 7.0 (Bernardino et al., 2011). Moreover, xerogel microparticles could be doped with magnetite, an approach that significantly eased the recovery of the immobilized biocatalyst. The particulate biocatalyst was preferably used in non-stirred bioreactors, but under carefully selected stirring conditions, xerogel particles did not display noticeable abrasion due to shear. This was also noticed when the versatility of the immobilization technique was challenged by using inulinase immobilization and inulin hydrolysis as model system. Despite of the different reaction system and although no changes in the procedure were made, very positive results were obtained, regarding product yield and reusability of the xerogel biocatalysts, that compare favorably with data reported in the literature for enzymatic inulin hydrolysis, including those obtained by our group (Table 1).

CONCLUSIONS AND OUTLOOKS

The design of bioconversion systems with industrial relevance anchored in the use of enzymes immobilized in synthetic gels as catalysts is a field of research where our group has been actively involved. The methodologies used have proved to be quite versatile, since they could be successfully implemented in different reaction systems. In order to cope in a timely, cost-effective manner with the typical steps required for the basic characterization of the immobilized biocatalysts, viz. assessment of encapsulation efficiency, pH-activity and temperature-activity profiles, enzyme kinetics and stability, preference has been given to the use of miniaturized platforms, viz. microtiter plates, minireactors, that allow for parallelization and high throughput. Along with good encapsulation efficiency and minimization of mass transfer effects, particular care is given as to ensure that the immobilized biocatalysts designed are resilient, so that their re-use/continuous-use is made over significant time-span/consecutive batches. Accordingly, their use may be realistically considered as potentially cost effective. Notwithstanding efforts made so far, further research on these matters clearly involves an increasingly transversal approach, involving further complementary fields of research, such as materials science, computational methods or protein structure, so that a more clear picture of the behavior of these immobilized biocatalysts emerges.

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Pedro Fernandes obtained a PhD in Biotechnology from Technical University of Lisbon in 1999. His research interests have been focused in applied biocatalysis and most of his work has involved the use of immobilized enzymes and cells. He has authored or co-authored over 70 papers and several book chapters.

ARTICLE

QUICK-RELEASE SUBTILISIN IS GRANULATED IN ALGINATE MICROSPHERES FOR DETERGENT FORMULATION

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INTRODUCTION

Subtilisin is a bacterially fermented protease, manufactured in granular form for use in powdered laundry detergents. Key objectives in granulation are (1) to ensure enzymatic stability during storage in detergents; (2) to provide dust-free particles safe for handling during manufacturing and use; and (3) to disintegrate and rapidly release active enzyme during a wash cycle. A collaboration was established between Queen's University and Genencor International (presently DuPont Industrial Biosciences), toward the development of a granulation method for quick-release subtilisin. In this report, two different methods of encapsulation will be described, and some experimental results presented, describing the characteristics of the granulated enzyme.

EMULSIFICATION/GELATION METHOD

Subtilisin was granulated by emulsification/internal gelation of alginate polymer (details provided in Liu et al., 2005; Chan et al., 2008). Alginate sol at 3%, containing subtilisin, ultrafine $CaCO_3$ and other additives was emulsified in canola oil at ratio of 1/4 and mixed in a vessel with impeller and baffles for 15 min to stabilize the emulsion. Emulsified droplets were gelled by rapid pH adjustment with acetic acid in oil, to reduce pH from 7.5 to 6.5 (figure 1). After 2 min continued mixing, the suspension was filtered to separate microspheres from the suspension, then washed several times with acetone, forming dry granules as illustrated in figure 1. Granules were spherical, and about 500 μm in diameter, although the diameter could be readily adjusted by controlling the emulsion conditions, such as through varying mixing, or through use of surfactant such Span 80.

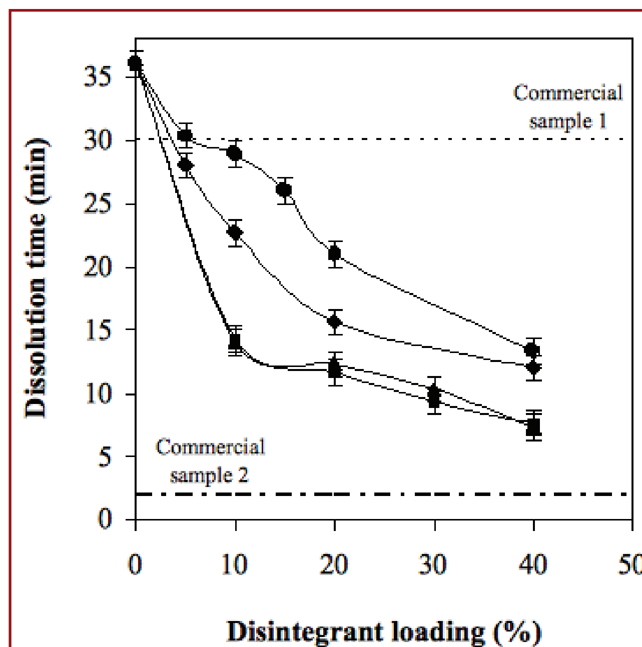

Figure 2: Subtilisin granule dissolution times, with increasing disintegrant concentration: MCC (■), PVA (▲), sucrose (◆), and starch (●). Granules also contained 10% TiO2 for color. Data is compared to that of 2 commercial granule samples.

The payload was 86 mg subtilisin/g granules and enzyme yields were approximately 90%. The payload could be increased to approaching 400 mg/g, but at the expense of encapsulation yield. Dramatic increases in yield could be achieved even at the highest payload, when higher molecular weight alginates were used.

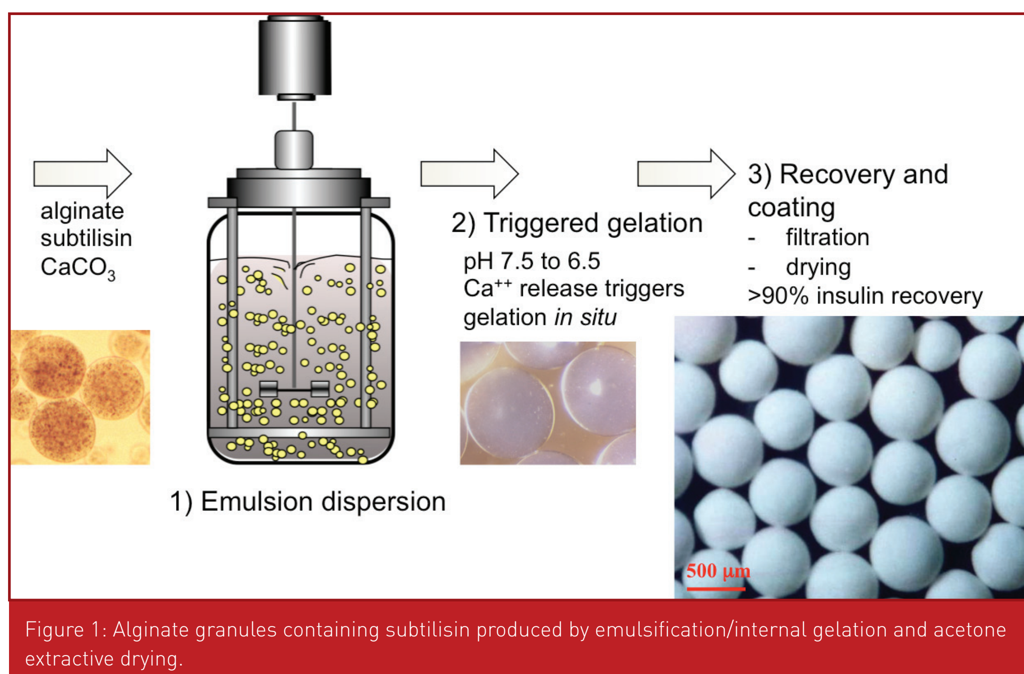

Figure 1: Alginate granules containing subtilisin produced by emulsification/internal gelation and acetone extractive drying.

Granulated subtilisin showed much higher temperature resistance than free enzyme. For example, over 80% of the activity remained after 180 min at 70oC, while 20% free subtilisin remained active after 5 min at the same temperature. The granulation matrix was an effective thermal insulator. In terms of shelf life, over 50% of granulated enzyme activity was retained after 250 days at room temperature.

Since granules are designed to release subtilisin in a wash, disintegrants were added to promote enzyme release. Granules were formulated with additives microcrystaline cellulose

(MCC), polyvinyl alcohol (PVA), sucrose or starch at levels up to 40% of the granule mass. Figure 2 shows dissolution times in a wash simulation for subtilisin granules containing varying amounts of additives. Dissolution times decreased with increasing disintegrant concentration, with all providing lower dissolution times than commercial sample 1 and longer dissolution times than sample 2. A dissolution time under 15 min is a desirable objective, and all disintegrants achieved that objective at concentrations under 40%. The most rapid release kinetics were observed with MCC and PVA as disintegrants.

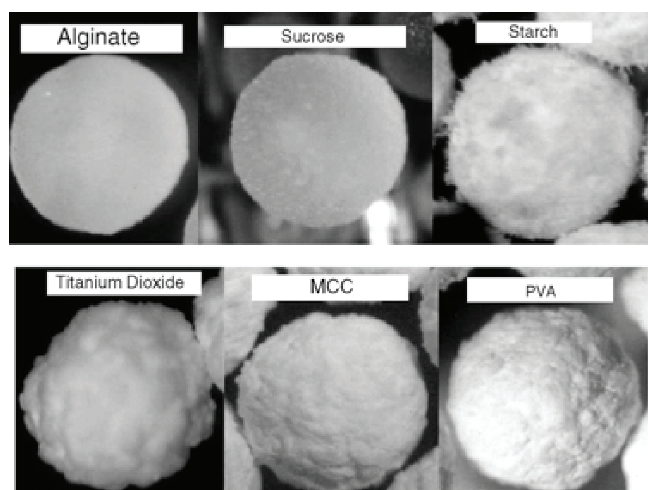

Figure 3: Granules consisting of 3% alginate alone or with 30% filler additives as labelled or 20% TiO_2. The granules shown have a diameter of approximately 700 µm.

Attrition resistance is an important parameter used to predict potential dusting problems involving enzyme granules under formulation and handling conditions. Granules with additives other than starch demonstrated greater attrition resistance, than two commercial enzyme granule samples. Granules containing starch showed less attrition resistance, likely do the effect of starch causing a fluffy attrition prone surface to the granule as seen in figure 3. Granules containing no disintegrant were fully intact and dust free after 21 min repeated impact test. In general then, higher additive levels resulted in reduced attrition resistance which is undesirable, but also resulted in decreased dissolution time, which is desirable.

Overall, MCC as a filler showed the best combination of promoting rapid granule dissolution, while having the least effect on dusting (attrition) property, where 93% of the granules remained intact after 21 min of repeated impact test.

Alginates with guluronic G-content greater than 46% showed exceptional impact resistance (over 80% remaining intact after 20 min repeated impact), with impact resistance decreasing dramatically at G-content of 37%. G-content did not affect dissolution rates of the granules. Likewise replacing Ca^{++} as the gelling ion with barium or ferric chloride did not affect granule impact resistance or dissolution time.

An optimal formulation was then prepared consisting of 3% Algogel alginate, 10% TiO_2, 40% MCC and 3% enzyme. The time course of enzyme release into a wash solution consisting of 1% Gessy Lever®, is illustrated in figure 4. More than 50% enzyme was released after 30 sec, and 90% after 3 min.

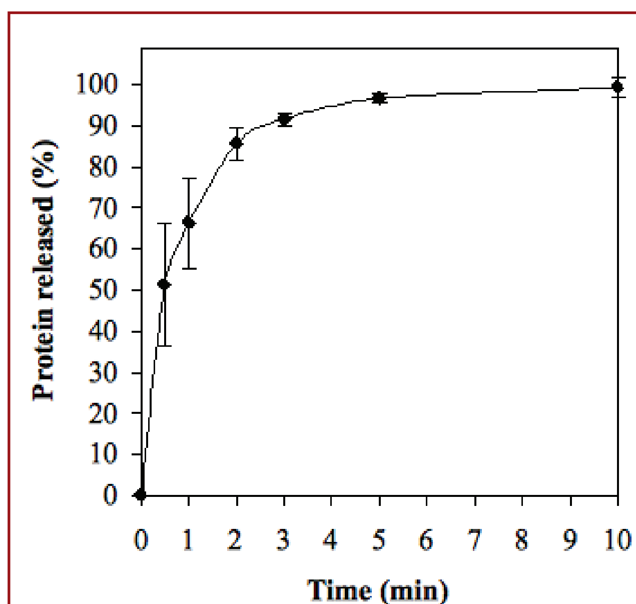

Figure 4: Subtilisin release profile from dry granules into detergent solution.

NEW ABSORPTIVE GRANULATION METHOD

An alternative approach to subtilisin encapsulation for detergent application was developed. Preformed alginate microbeads or dry granules were formulated as described above, then loaded with active subtilisin by immersion of the beads or granules into enzyme concentrate, in a method termed absorptive encapsulation and granulation. Such a technology platform could be potentially applied to other proteins and bioactives. Such an approach offers several advantages:

- labile encapsulates are not exposed to granule formulation conditions

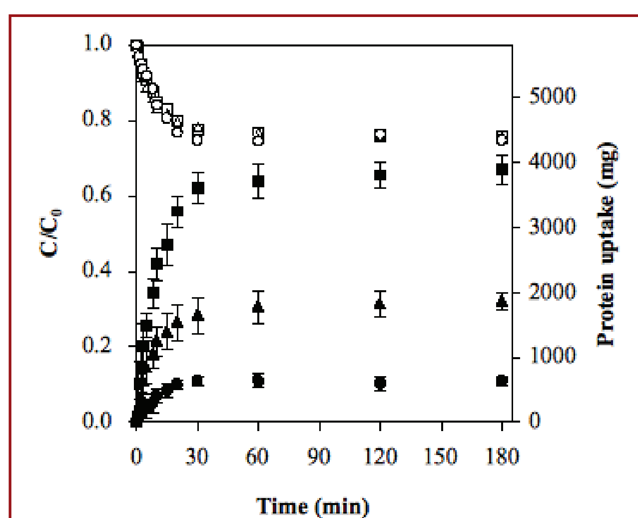

Figure 5: Absorption of subtilisin by 1.5% alginate (Algogel) beads in 50 (●), 150 (▲), and 300 mg protein/mL (■) enzyme concentrate (left side ordinate; open symbols). C is the residual subtilisin in supernatant, relative to the initial concentration, C0. The corresponding bead loading concentration was calculated from the concentration change in the loading solution (right side ordinate; closed symbols).

- «blank» microbeads or granules can be produced in large scale, sized then stored for subsequent loading
- particle size control is cost effective since fines or oversized blanks can be rejected or recycled without loss of costly active
- large batches to produce blanks is more cost effective
- subsequent loading can readily be accomplished in smaller, customized batches
- variety of natural or synthetic polymers can be used or blended to produce blanks providing wide range of properties for individual applications.

Blanks were produced using the formulation method described in the previous section. An example of absorptive encapsulation of subtilisin into alginate blanks is illustrated in figure 5. Half the subtilisin is absorbed within 8 min, and equilibrium achieved after about 60 min. Higher supernatant concentrations increased protein loading into blank beads. Subtilisins have molecular weights of around 30 kDa, and thus are of a size that would readily diffuse in or out of an alginate bead. Once loaded, the beads were separated by filtration, and dried by acetone extractive drying, with protein activity yields approaching 95%. Protein payloads achieved in 50, 150 and 300 mg/mL enzyme concentrate were 305, 552 and 717 mg/g granules respectively, with activity essentially fully retained. Protein payloads were approximately the same, irrespective of the alginate G-content.

In comparison to more conventional enzyme encapsulation approaches, absorption by immersing preformed granules into the enzyme concentrate can be considered a rapid method, considering that close to 90% of the protein loading was achieved in the first 5 min. In addition, high payloads can be achieved, again in comparison to alternative encapsulation approaches. For example, with granules made from 3% S170 alginate, the protein payload was 541 mg/g granules and subtilisin activity was 33,353 IU/g granules. Thus over half the mass of the granules consists of active enzyme. Rapid protein uptake was mainly due to granule rehydration in the concentrate.

Payload could be readily controlled by varying the enzyme concentration in the concentrate. For example, granules formulated from 1.5% alginate, loaded in 50, 150 and 300 mg protein/mL concentrate solutions, resulted in final granule payloads of 190, 380 and 540 mg/g, with essentially full activity retention.

SUMMARY AND CONCLUSION

Active subtilisin was granulated in alginate by emulsification of alginate sol, followed by triggered internal gelation and acetone extractive drying. Granules were discrete, spherical and smooth. Diameter was adjustable through control of emulsification parameters, but granules having size of 500 Ðm were considered desirable, to match the size of detergent granules. Encapsulation yields were high, and granulated subtilisin largely retained full enzyme activity.

Alginate granules containing subtilisin but no other filler components were fully resistant to abrasion in an extended and repeated impact test, but filler components required to promote dispersibility in detergent solution resulted in increased susceptibility to abrasion, and dusting potential. Increased amount of dispersants in the granules promoted granule dissolution kinetics, but at the same time, contributed to abrasion susceptibility and dusting potential. Granule abrasion/attrition resistance was enhanced at higher alginate concentration and higher G-content alginate, while dissolution rate was improved through appropriate selection of filler additives and loading levels. An optimal formulation providing high level of mechanical strength and rapid protein release in detergent solution was obtained with alginate/microcrystaline cellulose granules containing subtilisin and TiO_2 as colorant.

It is proposed that «blank» alginate granules or beads be prepared as a technology platform for the subsequent loading of subtilisin, and potentially other enzymes and bioactives. This approach was demonstrated by immersing blank alginate beads and granules into enzyme concentrate for loading, followed by acetone extractive drying. Full retention of subtilisin enzymatic activity was achieved, and enzyme loading was readily controllable, to levels as high as over 50% of the granule mass, levels that are considerably higher than most conventional granulation and encapsulation technologies. The formulation protocol is simple, achieves high encapsulation yields, short formulation times, and the ability to tailor the properties of the resulting beads or granules through the use of natural or synthetic polymers or blends.

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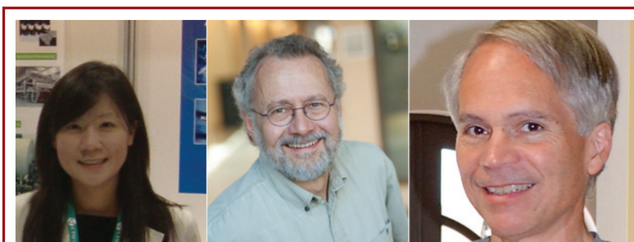

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Ron Neufeld is a Professor of Chemical Engineering at Queen's University, Kingston, Canada. All have been participants at BRG meetings, and this collaboration was the result of networking opportunities made possible through the BRG.

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NANOFAR: European Erasmus Mundus Doctorate in Nanomedicine and pharmaceutical innovation

NanoFar is an Erasmus Mundus Joint Doctorate programme selected in 2011 by EACEA. This European programme is coordinated by the University of Angers (France), and involves the Universities of Nantes (France), Liège and Louvain (Belgium), Nottingham (United Kingdom) and Santiago de Compostela (Spain). NanoFar is a three-year PhD thesis, co-supervised by partners in two different countries and including a one-year mobility period in a partner country. It aims to train the best students in the field of Nanomedicine at the doctoral level. The Doctorate course has 120 ECTS credits dedicated to laboratory based research, and 60 ECTS credits for training activities, including 3 mandatory summer schools. Nine Erasmus Mundus Doctorate fellowships (covering tuition fees, participation costs, travel expenses, some household expenses contribution and a monthly allowance) will be offered in 2014 by the EMJD NanoFar consortium to students from all over the world. Additionally, PhD fellowships locally funded will be available.

Selection process is open until 31th January 2014.

Information www.erasmusmundus-nanofar.eu

Open position

Polymer chemist Postdoc position
Polymer Institute SAS, Bratislava, Slovakia

A post-doc/research scientist position for the pulsed-laser polymerization combined with size-exclusion chromatography, PLP-SEC, and related studies is available in Prof. Lacik's kinetics group at the Polymer Institute SAS in Bratislava starting from next year. The topic is related to ongoing activities predominantly into propagation kinetics of homo and copolymerization for neutral and charged water-soluble monomers in aqueous and non-aqueous solvents. In the last years, major progress in understanding this area of polymer chemistry has been made. The successful applicant is expected to contribute to further achievements in this field. This work has been performed in cooperation with universities in Europe and Canada and an industrial partner. The outcomes have significant implications to mechanism and kinetics of polymerization as well as to modeling of industrial processes. The applicant should demonstrate a strong knowledge in macromolecular chemistry and interest to enter the field of radical polymerization kinetics of water-soluble monomers. The preference will be given to an applicant with experience in pulsed-laser initiated polymerization and size-exclusion chromatography, although this condition is not obligatory. A good level of spoken and written English is required. Interested applicant should send the email to Prof. Igor Lacik with professional CV, motivation letter, contact details and the contact to an academic referee.

Contact igor.lacik@savba.sk

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ARTICLE

ENZYME STRUCTURE AFTER ENCAPSULATION INTO MICRO AND NANOPARTICLES

Flávia Sousa, Pedro Fonte and Bruno Sarmento - CESPU, IINFACTS - Gandra-PRD, Portugal

INTRODUCTION

Protein biomolecules such as enzymes are well known either by their therapeutic potential in various diseases or by their use in food industry. However, they have some limitations such as: high molecular weight, hydrophilic character, short half-life, high rate of elimination and limited ability to cross membranes. To overcome these limitations, the encapsulation of enzymes on micro and nanocarriers have been used in order to improve their, stability and bioavailability (Yadav, Kumari, and Yadav 2011). To encapsulate enzymes into micro and nanocarriers, it is necessary to choose the ideal method of encapsulation to avoid protein instability during formulation. The majority of encapsulation methods used involve protein exposition to aqueous/organic interfaces, mechanical stress and dehydration. This represents a problem because protein aggregation, inactivation and denaturation of encapsulated enzymes frequently occur. When this occurs and protein-loaded carriers are administrated, reactions of immunogenicity can take place (Pérez, De Jesús, and Griebenow 2002).These alterations of proteins will be reflected in changes in the structure of the enzyme, therefore it is fundamental to determine the enzyme structure after encapsulation. To evaluate protein structure several methods are used such as: Fourier transform *infrared* spectroscopy (FTIR), *circular dichroism* (CD), and Fluorescence Spectroscopy and Nuclear Magnetic Resonance (NMR) spectroscopy.

This review will briefly discuss the techniques used to encapsulate enzymes, as well as the structure of the enzymes after encapsulation into micro and nanoparticles, in order to understand how the encapsulation process may interfere with enzymes structure.

ENZYME ENCAPSULATION METHODS

To encapsulate enzymes, the most commonly used carriers are polymeric microspheres (Castellanos et al. 2002), liposomes (Rodriguez-Nogales and Delgadillo 2005), lipid vesicles (Tan et al. 2010) and nanotubes (Park et al. 2012). Liposomes can be prepared through different preparation methods such as lipid film hydration, high-intensity ultrasonication, high-pressure homogenization, extrusion, membrane homogenization, reverse-phase evaporation, detergent depletion (Park, Yoon, and Kim 2010). However, not all of these methods are used to encapsulate enzymes into liposomes since some of them may denature these biomolecules.

The most frequently used method to encapsulate enzymes into liposomes is the lipid film hydration method followed by extrusion. However, some parameters such as phospholipid type and concentration, buffer salt, pH and ionic strength, liposome size, enzyme concentration and molecular weight of enzyme and electrostatic interactions between the phospholipid and the enzyme, can influence the efficacy of protein encapsulation (Hwang et al. 2012). The influence of such parameters on enzyme encapsulation have been studied (Hwang et al. 2012). Using trypsin as a model, the encapsulation efficacy increased linearly with the phospholipid concentration and the liposome size, which means that these parameters can be optimized in order to improve enzyme encapsulation. In addition, the encapsulation yield increased with the reduction of ionic strength since the lower ionic strength reduces the solubility of the enzyme and promotes hydrophobic interactions between protein and lipid bilayer. This theory was also confirmed since with the increase of ionic strength, the encapsulation yield decreased (see Figure 1).

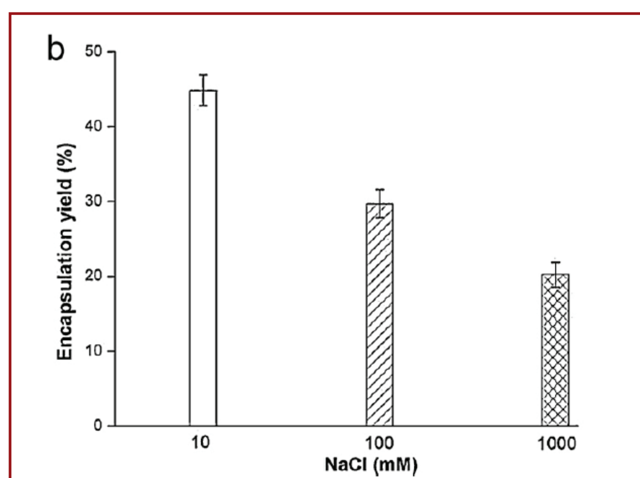

Figure 1. Effect of NaCl concentration on trypsin encapsulation yield (Hwang et al. 2012).

In another study it was shown that it is possible to encapsulate β-galactosidase in liposomes by the dehydration-rehydration method (Rodriguez-Nogales and Delgadillo 2005). This method presents some advantages when compared to other methods because it offers a larger encapsulation efficacy and it is an easy method to scale-up.

The most used method in encapsulation of enzyme-loaded bioerodible polymers (such as (lactic-co-glycolic) acid - PLGA), is water-in-oil-in-water (w/o/w) emulsion technique. W/O/W emulsion consists in small water droplets within solid lipid particles and hydrophilic drugs are dissolved into the aqueous phase (see Figure 2a). However, the formation of the first water-ion-oil (w/o) emulsion is a considerable cause of protein's inactivation and aggregation because of the exposition of the drug protein in the aqueous/organic solvent (normally methylene chloride) interface (Pérez, De Jesús, and Griebenow 2002). When proteins are in this interface the adsorption of protein in the CH_2Cl_2 interface occurs, due to exposition of the hydrophobic domains in proteins surface, which may interferes with protein structure. In addition, the mechanical forces used in the creation of the w/o emulsion may also lead to the loss of protein structure. To overcome this, alternative methods of encapsulation in

order to avoid the aqueous/organic interface formation have been studied, such as the solid-in-oil-in-water (s/o/w) technique (Castellanos et al. 2002). S/O/W dispersions consist in hydrophilic drugs dispersed within the oil phase that, in turn, are dispersed in an external water phase (see Figure 2b) (Kukizaki 2009). However, this technique also presents some limitations, namely its difficulty to ensure the homogeneous distribution of enzyme powder particles in the microspheres or nanospheres.

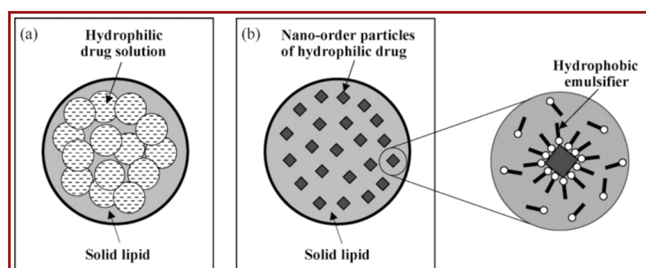

Figure 2. Schematic illustration of the drug-loaded solid lipid microcapsules prepared from (a) w/o/w emulsion and (b) s/o/w dispersion (Kukizaki 2009).

It is also possible to encapsulate enzymes in hydrogel systems. These hydrogel systems are composed by alginate or chitosan and are very frequently used in protein delivery systems since they have a consistency similar to natural tissue and high water content. Briones et al. studied the use of a chitosan-carrageenan polyelectrolyte complex as carrier of glucose oxidase (GOD) (Briones and Sato 2010). It is possible to use the complex chitosan-carrageenan because electrostatic interactions occur between the positive charge of amine groups in chitosan and the negative charge of sulphate groups in carrageenan. In this study as GOD was negatively charged it was possible to form a complex with chitosan and, as shown in Figure 3, the entrapment efficacy was elevated.

Enzyme structure after encapsulation

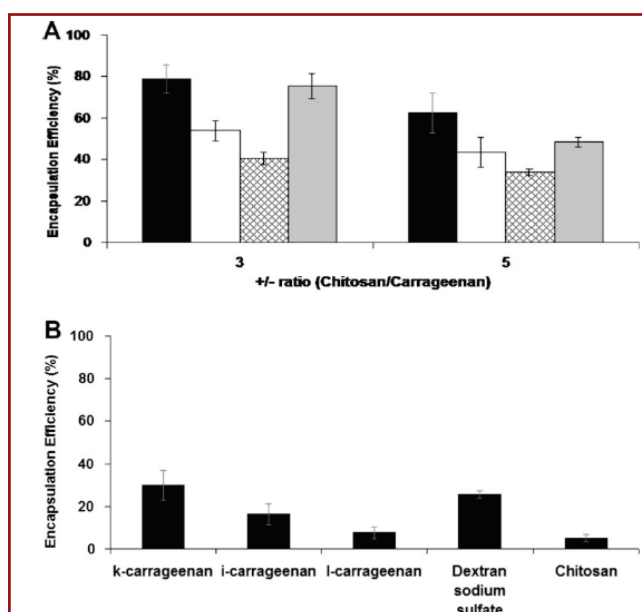

Figure 3. Encapsulation efficiency (%) of a chitosan/ carrageenan complex (A) and single polymers (B) to encapsulate GOD (Briones and Sato 2010).

During the formulation process of enzyme-loaded micro and nanoparticles, it is necessary to choose the best method to encapsulate the enzyme selected in order to avoid the problems associated with instability, aggregation and inactivation of these biomolecules. Therefore, it is important to evaluate the protein structure before and after encapsulation (van de Weert et al. 2000; Castellanos et al. 2002). As mentioned, the structure perturbations of protein may be different depending on the carrier used for encapsulation and on the encapsulation process.

Polymeric matrices

In order to determine the secondary structure of enzymes encapsulated into polymeric matrices it is necessary to extract them. However this process can cause conformational alterations in the protein (van de Weert et al. 2000). Therefore, the ideal method to determinate the secondary structure of proteins should be non-destructive analyses such as FTIR, unlike others methods such as CD and NMR.

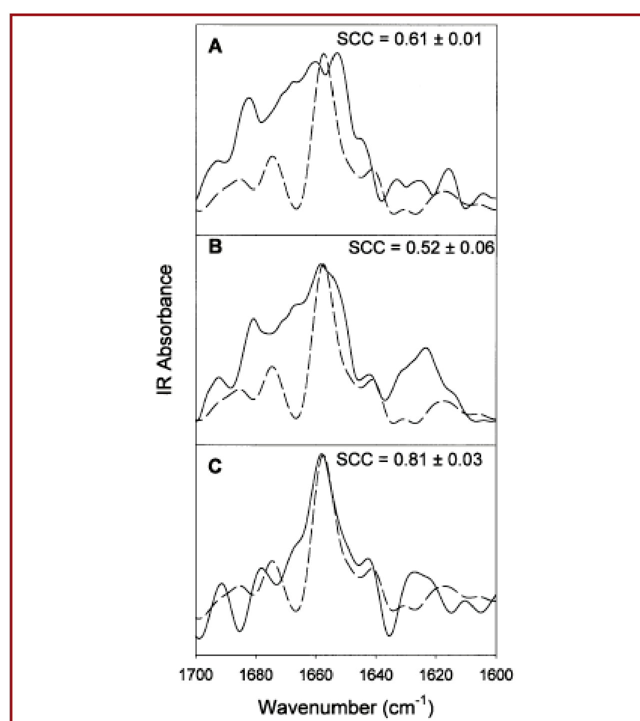

Figure 4. Inverted second derivate amide I infrared spectra of lysozyme in aqueous solution at pH 5.1 (dashed line), lysozyme encapsulated without additives (A-full line), lysozyme encapsulated with sucrose (B-full line) and with lactulose (C-full line) (Pérez, De Jesús, and Griebenow 2002).

In a previous study, lysozyme structure after encapsulation in microspheres of PLGA (through w/o/w emulsion technique) in presence of several excipients was studied, in order to understand if the used excipients interfered in the lysozyme stability (Pérez, De Jesús, and Griebenow 2002). After encapsulation without stabilizing excipients, FTIR data showed several changes in the enzyme structure (see Figure 4a), mainly because α-helix content decreased from 36 to 26%. When this enzyme was encapsulated with sucrose and trehalose secondary structural alterations were more pronounced (see Figure 4B). The use of lactulose and lactose effectively stabilized lysozyme at the w/o interface (see Figure 4C). Overall, this study demonstrated that the change of the secondary structure of lysozyme can be pre-

vented by using efficient excipients during encapsulation.

In another study, the secondary structure of ϒ-chymotrypsin encapsulated into PLGA microspheres by s/o/w technique was assessed (Castellanos et al. 2002). It was concluded that encapsulation of ϒ-chymotrypsin into PLGA microspheres by s/o/w technique caused protein aggregation, secondary structure perturbations and loss of bioactivity. Furthermore, in vitro release experiments showed an incomplete release due to the protein aggregates formed upon encapsulation.

Liposomes

The effect of pH on superoxide dismutase (SOD) encapsulation into liposomes, enzyme structure and its enzymatic activity was investigated (Corvo et al. 2002). Protein structure was evaluated through CD. Interestingly, SOD secondary structure did not change at pH 3.3 and 5.6 unlike to tertiary structure (see Figure 5). However, data obtained by CD did not show correlation with enzymatic activity because with pH increased, SOD activity also increased despite the fact that tertiary structure kept altered. This probably occurs because the tertiary structure perturbations don't influence the active center of SOD instantly. However, this fact needs to be considered because tertiary structure is fundamental for pharmacokinetic profile or immunogenicity of biomolecules such as enzymes.

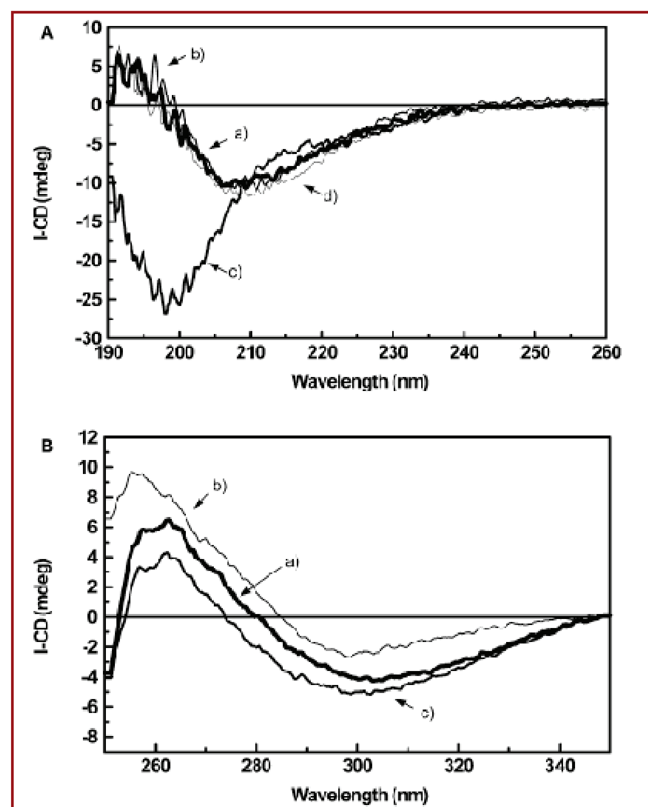

Figure 5. CD spectra of SOD encapsulated. (A) Secondary structure of SOD: (a) pH 5.6, (b) pH 3.3, (c) pH 1.5, (d) pH 1.5Ð5.6 (solution titrated from pH 1.5 to 5.6). (B) Tertiary structure information: (a) pH 5.6, (b) pH 3.3, (c) pH 3.3Ð5.6 (Corvo et al. 2002).

Nanogels

Enzymes may also be loaded into nanogels. These carriers are particles at the nanometer range with a large surface area and high water content, which makes them good carriers of biomolecules (Oh, Lee, and Park 2009). Some studies have been made in order to prove the encapsulation efficacy of nanogels. In a previous study, the encapsulation efficacy of asparaginase in nanogels as well as the secondary structure of this enzyme before and after encapsulation was assessed (Steinhilber et al. 2013). It was showed that the encapsulation efficacy was about 100% and no significant changes in the secondary structure of asparaginase after encapsulation was observed.

CONCLUSION

It is necessary to evaluate enzyme structure after encapsulation because if the enzyme structure is altered , it may have no biological activity or even cause immunogenicity and toxicity problems when released from the carriers. This is a major problem because despite having high encapsulation efficacy, the enzymes structure may have changed and therefore, will not have the desired therapeutic effect. The encapsulation method, parameters and excipients of formulation or even interactions between carriers and loaded enzymes are sources of enzyme instability and have to be considered when enzyme encapsulation is been used. Just a few studies which evaluate the structure of enzymes after encapsulation and its correlation with enzymes bioactivity are available, thus more studies regarding this issues are necessary.

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Job request

Ankur Goel

Looking for position in the field of food ingredient encapsulation

Affiliation Research Assistant (Fundamental & Applied Research – McCain Foods)

Ankur Goel is currently working in the "Fundamental & Applied Research" group at McCain Foods Canada. He obtained his Bachelors in Biotechnology from Amity University (India) and Masters in Food Science from McGill University (Montreal, Canada). During his masters degree, he worked on optimization of method for the characterization of nano encapsulation of bioactive molecules (protein/ polysaccharides) as ingredients, using ultra sonication.

Currently at McCain Foods, he has experience in the field of food science, freezing technology, ingredients/ coatings, process optimization, unit operation studies, new product development, enzyme application, nutrition and multiple R&D project management.

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Job request

Pérignon Carole, PhD

Looking for a job in R&D in the field of food, cosmetic or chemistry

Master 1 in Chemistry and Graduated in Food Sciences at Ecole Nationale Supérieure d'Agronomie et des Industries Agroalimentaires in Nancy, I did my PhD in chemical reactions in microencapsulation under the direction of Dr. Poncelet at Oniris in Nantes. This multidiscipline formation gave me broad Knowledge over various fields such as food formulation, polymer physico-chemistry and encapsulation technologies. I am experienced in project management and in team work. Perseverance, organization and flexibility are my qualities when leading a project.

I am looking for an opportunity as a project manager in R&D in food, cosmetic or chemistry fields in France.

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ARTICLE

SOL-GEL TECHNIQUES FOR ENCAPSULATION OF ENZYMES

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Introduction

A large part of formulated peptides and proteins, e.g., enzymes used as food ingredients, are formulated in a liquid form. Nevertheless, there are reasons such as stability and reduced transportation costs, which require formulation in a solid form. The problem of using powdered proteins is that people can develop allergic reactions when exposed to them for long periods of time or to high concentrations. Among the different methods for enzyme immobilization, encapsulation is of particular importance because of the simplicity of preparation, the biomolecule freedom and safe products which can be obtained. The encapsulation process is based on the entrapment of the biomolecule in a polymer matrix and there is no covalent association between the network and the biomolecule. This entrapment restricts rotation and unfolding movements but allows substrate recognition and binding as well as catalysis. In this contribution the sol-gel techniques for enzyme encapsulation are outlined. The sol-gel technology is based on the formation of silica matrices of metal or semi-metal oxides through the aqueous processing of hydrolytically labile precursors. Hydrolysis and condensation then lead to the formation of a porous silica network in which biomolecules remain trapped.

Methods

Encapsulation via Alkoxide pathway

As indicated by reactions of the Type 1 (Figure 1) and Type 2 (Figure 2), Alkoxide pathway of Sol-gel processing is based on the ability to form metal-oxide, silica, and organosiloxane matrices of defined porosity by the reaction of organic precursors at room temperature. Silicon alkoxides are characterized by the presence of Si-O polar covalent bonds, so in the first step, one or two metal alkoxide precursors (usually, tetramethoxysilane [TMOS; $Si(OCH_3)_4$] and/or methyltrimethoxysilane[MTMOS; $H_3Si(OCH_3)_3$] are hydrolyzed by reaction of type (1), which occurs in the presence of water at acid pH, leads to the replacement of OR ligands by OH ones and result in the formation of silanol (Si-OH) groups.

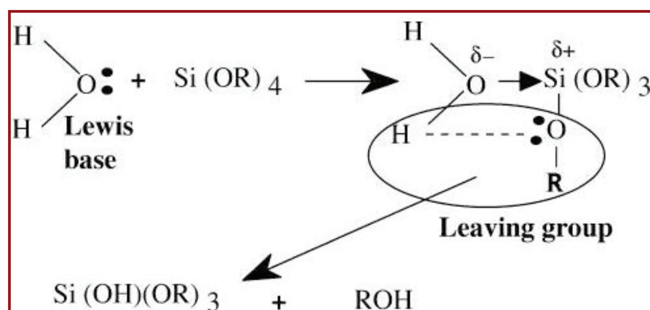


Figure 1: Reaction pathway for Type 1 reactions.

Materials

Tetramethoxysilane (TMOS; Sigma, St. Louis, MO), Methyltrimethoxysilane (MTMOS; Sigma), 1 mM HCl, Polyethylene glycol (PEG) with average mol. wt. 600 (PEG 600; Sigma), enzyme to be immobilized. Screen-printed graphite electrodes (BIOMEM Group, Université de Perpignan, France). Refrigerator or cold chamber.

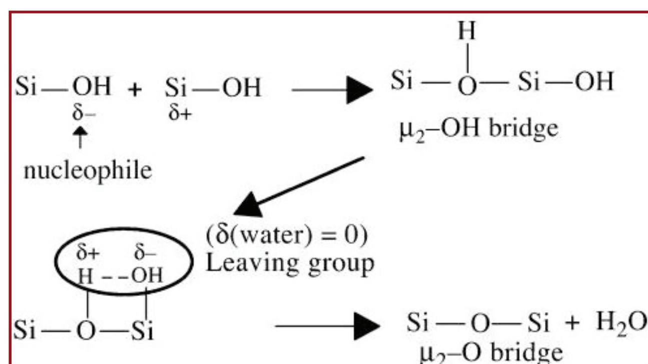


Figure 2: Reaction pathway for Type 2 reactions.

Method

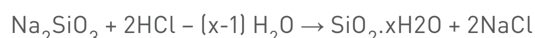
1. Mix the reactant solutions TMOS, MTMOS, H_2O, HCl (1 mM) and PEG600 in the convenient ratio in a 1.5-mL Eppendorf tube using automatic pipets
   Suggested ratios are shown in Table 1. However, other ratios, as well as other precursors, can be used. It should be taken into account that the higher the TMOS proportion, the faster the subsequent polymerization process.
2. Cap the Eppendorf tube and sonicate for 15 min to homogenize the mixture.
3. Store the Eppendorf tube at 4°C overnight to allow hydrolysis of the precursors.
   Hydrolysis time depends upon each case and should be optimized.
4. Dissolve the enzyme in a basic buffer using Milli-Q water.
   At basic pH, the condensation is favored. The appropriate basic pH will depend on the enzyme performance (not too high to inactivate it). The buffer composition is not particularly important. However, $(NH_4)2SO_4$ should not be used to avoid precipitation.
5. Mix the sol solution with the enzymatic one at a 50:50 ratio in a 1.5-mL Eppendorf tube, using automatic pipets to start the condensation.
   The 50:50 ratio is suggested. However, other ratios can be used.
   Controls are usually performed without enzyme but using the basic buffer.
   Sometimes the condensation occurs at faster rates. As a result the sol-gel starts to form before its deposition on the support surface. It is then convenient to carry out this step at 4°C (cold chamber).
6. Cap the Eppendorf tube and homogenize the mixture using a vortex mixer (see Note 6).
7. Spread the mixture on the support surface using an automatic pipet (see Notes 6–8).
   The volume of mixture deposited on the support surface depends on the particular interest, but it has never to spread out from the surface of interest.

Our experience is based on the sol-gel immobilization on screen-printed graphite electrodes. However, the sol-gel immobilization on other supports is also possible.

8. Dry the support surface for at least 36 h at 4°C.
   Drying time depends on each case and should be optimized. Desiccators with or without vacuum can also be used. This step can be also performed at room temperature. However, 4°C are preferred to maintain the enzyme activity.
9. Rinse the support surface with water prior utilization. While rinsing, carefully look to see if any desorption of the sol-gel from the surface support occurred.
   Buffer can also be used to rinse the support.

Encapsulation of Enzymes via aqueous pathway

In the Alkoxide pathway, even if alcohol is not added to the precursor solution, the hydrolysis and condensation of silicon alkoxides leads to the formation of alcohol as a by-product but the aqueous pathway does not produce such harmful by-products. Aqueous sol–gel chemistry is based on the condensation of solute precursors via pH modifications (Jolivet, 2000). According to reactions of the type (3), Ionic dissociation and hydrolysis occur when a silicate salt is dissolved in water leading to more or less protonated silicate species. In the case of sodium silicate a convenient commercial precursor for silica, a basic aqueous solution is obtained that contains and species [H_nSiO_4]$^{(4-n)-}$.



$$Na_2SiO_3 + 2HCl – (x-1)\ H_2O \rightarrow SiO_2.xH2O + 2NaCl$$

TMOS	10	10	10	2	2	2	5	5	5
MTMOS	10	10	10	20	20	20	10	10	10
H_2O	44	88	132	44	88	132	44	88	132
HCl	40	80	120	40	80	120	40	80	120
PEG600	4	4	4	4	4	4	4	4	4

Table 1: Precursor Mixture Compositions in µL (Final Volume: 108 mL).

Results

The alkoxysilane XSi(OR)3 carrying hydrophobic groups X are often more difficult to hydrolyze and condense than the alkoxides, except when the X function carries a functional group such as an amine or a carboxylate (Gill, 2001), because the organometallic Si-X bond cannot be hydrolyzed. TMOS, $Si(OMe)_4$ is currently used as a precursor rather than TEOS, $Si(OEt)_4$. Methanol is then liberated that has a polarity closer to water so, it is less harmful than ethanol. Nevertheless, even when the neat alkoxide is used as a precursor, concentrations of methanol up to 8 M could be reached via the fall hydrolysis of tetramethoxysilane, such an amount of alcohol can be harmful for enzymes and cells. It must therefore be eliminated before adding biomolecules. Hydrolysis is performed in the presence of a large excess of water, using hydrolysis ratios up to released alcohol is then highly diluted, gelation occurs almost entirely in water and xerogels with surface areas in excess of are obtained (Conroy, 2000). However silicon alkoxide precursors are usually not fully hydrolyzed during the first acid-catalyzed step, alcohol can still be released during condensation and ageing in the presence of the immobilized species. In the so-called Biosil process, hydrolysis is performed in the gas phase so that alcohol elimination is much easier (Cappelletti, 1999;

Carturan, 2001).With alkoxisilanes, a problem is that only a limited percentage of these precursors can be added before reaching a solubility limit. Nevertheless, this can be overcome by mixing these components very vigorously in a vortex mixer, or by sonication.

Conclusion

Natural and synthetic polymers such as polysaccharides, polyacrylamides or alginates are currently used for enzyme immobilization via covalent binding or entrapment. Enzymes are grafted onto the surface of silica gels, but this does not bring any real improvement and such a process can also be performed with porous glasses. Therefore most research work deals with the encapsulation of enzymes within a silica gel.

With sol–gel encapsulation enzymes are only physically trapped, without covalent bonding, within the hydrophilic aqueous environment provided by the silica matrix. Therefore most enzymes are not denatured by sol–gel encapsulation and their catalytic properties are similar to that of their water-soluble counterparts. Moreover, enzymes can even be stabilized in the gel. The silica matrix prevents their leaching and protects them against external reagents. In some cases, during encapsulation the silica network grows around the enzymes that remain trapped within pores tailored to their size.

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