Bioencapsulation Research Group

Bioencapsulation Innovations

September 2013

CONTENTS

EDITORIAL

XXI INTERNATIONAL CONFERENCE ON BIOENCAPSULATION

Berlin, Germany - August 28-30, 2013

The 21th International Conference on Bioencapsulation was very efficiently organised at Seminaris Campus Hotel of Berlin by Professor Stephan Drusch from Technological University of Berlin and Dr Thorsten Brandau from Brace GmbH.

The conference was a real success with regard to the quality of the oral and poster presentations, but also in the number of fruitful discussions which took place during the conference, and during the social dinner at the Brauhaus Lemke Brewery.

One hundred and seventy researchers coming from 33 different countries, including 47 industrials and 13 exhibitors. The organisers would like to thank in particular the exhibitors, as well as Symrise and P&G, for their contribution and support to the success of the conference. Fourty oral contributions and seventy six posters were presented during the conference. Their associated texts will be available soon on the BRG web site.

The scientific committee selected 5 orals and 5 posters for the student best contribution and all recieved prizes. The present issue compiles their texts. The conference and especially the best contribution prizes was dedicated to the memory of Dr Horst Dautzenberg, a great scientist and one of the most active members of the BRG (see page 6).

The participants were invited to nominate a candidate for the Poncelet Award sponsored by Procter and Gamble, and rewarding a person having strongly contributed to the innovation and development of the Bioencapsulation. This year the award was given to Professor Ronald J. Neufeld from Queen's University in Canada.

Ron has been one of the co-founders of the BRG. He is not only a leading scientist, he also has been a driving force for the BRG. He organized two international conferences on Bioencapsulation and several workshops. Each year, several of his students present outstanding research, and have won many prizes for the best contribution on the meeting. You will find Ron's recent work on page 4.
During the conference, the General Assembly of the association was been held. The minutes of this assembly are included in page 35.

Prof. Denis Poncelet
President of BRG

ARTICLE

IN VITRO SIMULATION OF *IN VIVO* PERFORMANCE OF ORAL DOSED NANOPARTICULATE INSULIN

Neufeld, R.J.

INTRODUCTION AND OBJECTIVES

At previous BRG meetings, we described an international collaborative research effort involving three outstanding PhD students who have now completed their programs (Drs. Catarina Pinto Reis, Bruno Sarmento and Camile Woitiski), three Master's students (Kristen Bowey, Kaitlin Reilly, Donya Golkaran) and the supervising team (Profs. Christiane Damgé, Université Louis Pasteur; António Ribeiro, Francisco Veiga, Rui Carvalho, University of Coimbra; Domingos Ferreira, University of Porto; and Ronald Neufeld, Queen's). The effort was directed toward the development of complex nanoparticulate multilayer polymeric structures for oral delivery of insulin. The most effective formulation involved an insulin-loaded alginate-dextran core, ionically complexed with a chitosan-PEG shell and finally with an albumin coat. The formulation reduced glycaemia in diabetic rats over a 24h period following a single oral administration, in a dose dependent manner. Pharmacological availability was by far the highest (42%) of any reported oral dosage form for insulin, and bioavailability was 30% relative to subcutaneous administration (Reis, 2008).

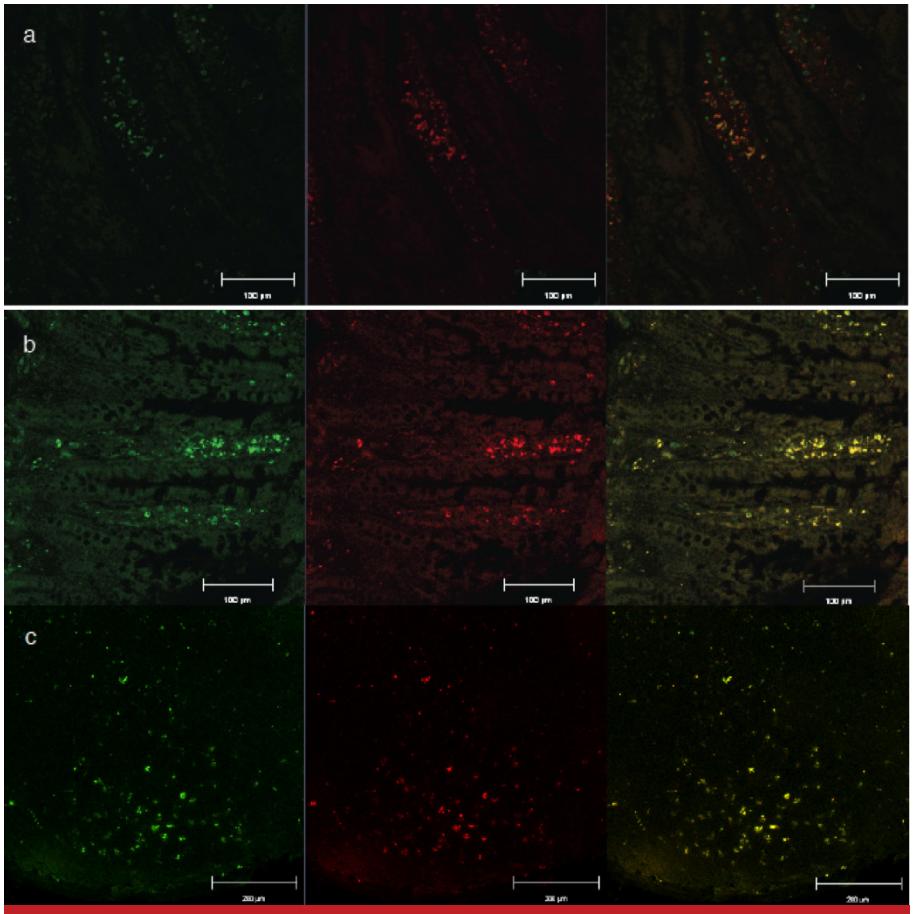

Figure 2: Cross-sections of intestinal mucosa of Wistar rats: (a) duodenum (scale bar 100 µm), (b) jejunum (scale bar 50 µm), and (c) ileal Peyer's patches (scale bar 200 µm) showing co-localization of nanoparticulate insulin by observing FITC-insulin (a1, b1, c1), RBITC-alginate (a2, b2, c2), and both in combination (a3, b3, c3). A corresponding 3D image (not shown) shows internalization of insulin nanoparticles (Woitiski, 2011).

Selection of polymeric components of the nanoplex required biocompatible materials, mucoadhesive properties (alginate, chitosan, PEG), ability to promote retention of entrapped insulin (alginate, dextran sulphate, chitosan), enable permeation through tight junctions between epithelial cells (chitosan, PEG), promote insulin and nanoparticle stability (PEG), and provide protection from gastrointestinal tract (GIT) proteases through use of a sacrificial coating of albumin. Alternative protein coats did not provide the protection from protease attack as did BSA.

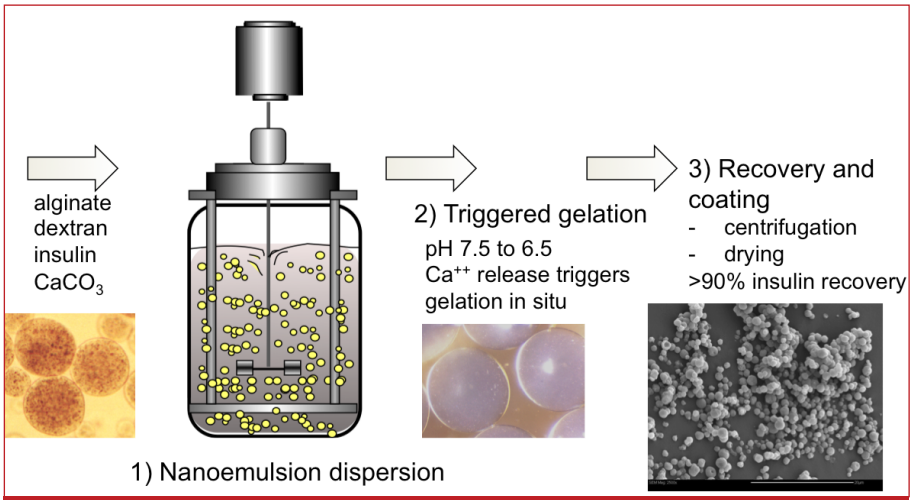

Figure 1: Method to produce insulin loaded nanoparticles by nanoemulsion dispersion, triggered in situ gelation.

Three methods were devised to formulate nanoplex structures. The first involved nanoemulsion dispersion of the core polymer components within a suitable oil phase as illustrated in

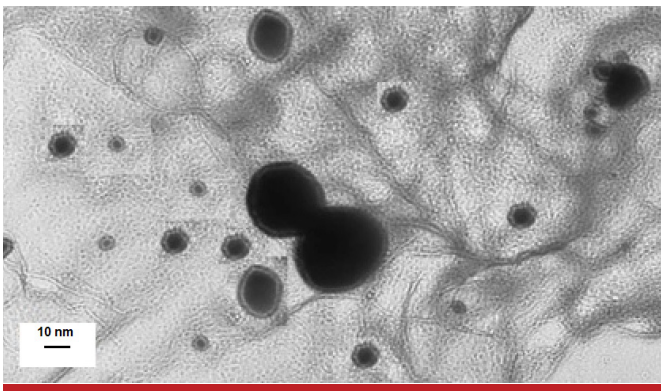

Figure 3: TEM of insulin nanoparticles

figure 1. Addition of ultrafine insoluble $CaCO_3$ to the core material enabled triggering of core gelation through release of Ca^{++} *in situ*, through gentle pH adjustment in a method termed «internal gelation». Particles were recovered (Pinto Reis, 2007) and subsequently coated in separate coating steps.

2013 Winner of Poncelet Award
For his contribution to development and innovation into bioencapsulation domain
Sponsored by Procter & Gamble

The second method involved Ca^{++}-mediated self-assembly of dilute alginate polymer forming pre-gel nuclei around insulin, followed by chitosan polyelectrolyte complex coating (Sarmento, 2007). The third method involved nanospray drying.

Nanoparticles and insulin were independently labelled and tracked through the GIT, and following internalization via the intestinal mucosa and enterocytes, into the mesenteric blood, thus through the portal-hepatic route of administration. Co-localization of the labels show that insulin is translocated along with the nanoparticle carriers likely through the transcellular route as illustrated in figure 2 (Woitiski, 2011).

In vitro simulations were conducted by measuring insulin nanoparticulate permeation through intestinal membrane models. Caco-2/HT29 cell monolayers more closely simulated nanoparticulate permeation through the animal model intestinal mucosa, showing the importance of mucus-secreting cells for nanoparticles constructed of mucoadhesive biomaterials. Improved and intimate contact between the nanoparticles and mucosal layer thus appear to promote nanoparticle permeation, certainly in comparison to insulin alone which was poorly absorbed (Woitiski, 2011). Chitosan as a coating polymer is known to open tight junctions between enterocytes, and the albumin coating was shown to protect insulin from proteolytic enzymes (Reis, 2008).

Detailed toxicological assessments were conducted following daily dosing of two different forms of nanoparticulate insulin for glycemic management in diabetic rats. Haematological, biochemical and urine-based assays, and organ and tissue histology show absence of toxicological effects, and no mortality was observed. Some observed effects were attributed to diabetes physiopathology, inducement or evolution status and thus not directly associated with oral administration of the nanoparticulate drug (Reis, 2008; Woitiski, 2012).

Since less than half of the insulin was accounted for in the *in vivo* trials conducted thus far, GI simulations were conducted in the study described below to examine factors affecting insulin nanoparticulate retention, release and stability. The work that is described below was conducted by the most recent graduate student completing her program, Donya Golkaran.

MATERIALS & METHODS

Insulin nanoparticles were prepared by mixing human recombinant insulin with 2% alginate solution containing 5% ultrafine calcium carbonate, and 0.75% dextran sulphate. The mixture was emulsified in paraffin oil at high speed, facilitated with 2.5% Span 80. After 15 min, gelation was induced by addition of paraffin oil containing acetic acid to adjust the pH from about 7.5 to 6.5. After separating particles from the dispersion, particle cores were coated in PEG-chitosan, then in albumin solutions. Details are provided in (Reis, 2008). Insulin assay was conducted by ELISA and HPLC, and particle sizing by Malvern Zetasizer.

Queen's University

RESULTS & DISCUSSION

Nanoparticles as described above are illustrated in the TEM image of figure 3, showing multilayered structures representing particle core and two polymer coatings. Mean diameter was as low as 10 nm, Zeta potential was -7 mV and insulin encapsulation efficiency was 85%.

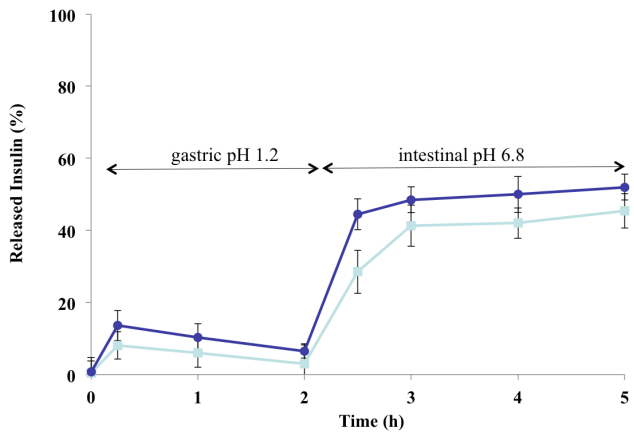

Figure 4: Insulin release profile from nanoparticles, monitored for 2h under simulated gastric condition followed by an additional 3h in simulated intestinal condition. Insulin release measured by ELISA in the presence (●) and absence (■) of pepsin (gastric) and trypsin (intestinal simulation).

A release profile of insulin in gastric (2h) followed by intestinal (3h) simulation is provided in figure 4, in presence of gastric and intestinal proteases and in the absence of proteases. Minimal release of insulin is observed in acidic condition, likely due to the collapsed and increasingly imper-

ARTICLE

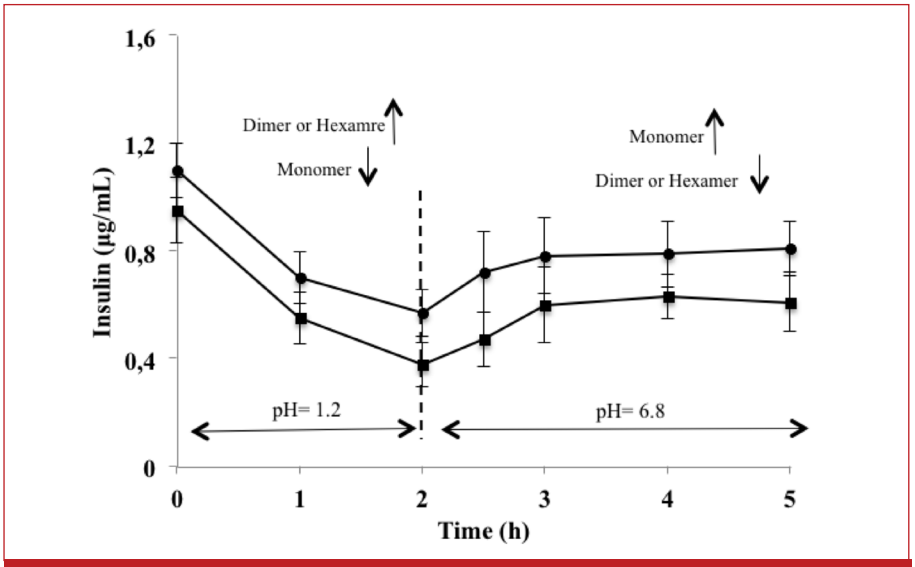

Figure 5: Insulin stability monitored by ELISA for 2h under simulated gastric condition followed by an additional 3h under simulated intestinal condition. Stability was tracked in the absence (●) and presence (■) of gastric and intestinal proteases. Acid condition results in conformational changes in insulin quaternary structure in the way that the amount of dimer and hexamer increased in comparison to monomer. Following the pH change to 6.8, the amount of monomer to dimer or hexamer increased.

meable structure of the core polymer alginate, followed by particle swelling in neutral pH medium initiating the release of insulin over the subsequent 3h period. Elevated release of insulin in presence of proteases may be due to removal of BSA coating, causing particles to become more permeable. Particle diameter was reduced by about 25% following addition to gastric (acidic) condition, then a doubling in particle diameter was observed following transition back to neutral pH conditions, explaining enhanced permeability to insulin.

At the end of the 5h simulation, particles were collected and dissolved in PBS containing EDTA. Released insulin was quantified as 54% of the initial loaded insulin from particles recovered from simulation without enzyme, and 46% from particles from simulation with proteases present. Thus all of the initial insulin is accounted for, with approximately half being released and half being retained by the particles. These values are rather similar to the value of 42% pharmacological availability reported for assays with rats (Reis, 2008). A simple interpretation is that 42% of the insulin is accounted for, relative to injectable insulin. The question then is whether it is the released fraction that represents available insulin when delivered close to the intestinal mucosal layer, or the retained fraction enabling uptake of particulate insulin as observed in cell based in vitro simulations, and in fluorescent tracking studies.

The effect of acid and protease enzymes on insulin stability was then examined in gastric followed by intestinal simulation as presented in figure 5. It is apparent that while only small amounts of insulin are released in acid medium, ELISA detectable insulin appears highly susceptible to both acid and pepsin. Insulin appears subsequently stable in intestinal simulation for an additional 3h, including in the presence of trypsin, and in fact an apparent increase in insulin concentration is evident. This experiment was repeated measuring insulin by HPLC detection as shown in figure 6 with quite different results. In this case, insulin appears stable in acid, protease rich medium, but appears to decompose in neutral medium, particularly in the presence of proteases. Insulin in buffer solution at neutral pH is stable over 5h, whether measured by ELISA or HPLC.

An explanation for these differences may be found in the behavior of insulin under different pH conditions. As identified in figures 5 and 6, insulin equilibrium tends toward a stable hexameric structure in acid condition in the presence of zinc, with the equilibrium shifting toward more susceptible monomeric form in neutral pH (Bryant, 1993). ELISA is based on monoclonal antibody binding to monomeric insulin, based on 1 or 2 insulin molecules potentially bound per antibody. It is likely then that higher order structures (dimers and hexamers) do not interact stoichiometrically with ELISA antibodies likely due to steric effects, thus the appearance of reduced insulin concentration as it forms hexameric structures in acid, and appearance of increased insulin concentration as

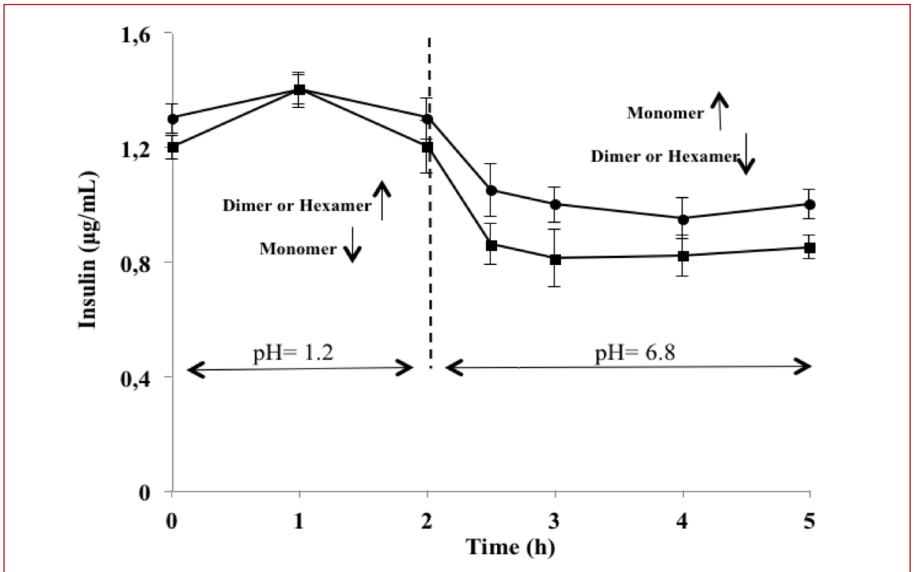

Figure 6: Insulin measureable by HPLC, 2h under simulated gastric condition followed by 3h under simulated intestinal condition in the absence (●) and presence of pepsin for gastric condition and trypsin for intestinal condition (■). Acid condition causes conformational changes in insulin quaternary structure in the way that the amount of dimer and hexamer increased in comparison to monomer. Following the pH change to 6.8, the amount of monomer to dimer or hexamer increased.

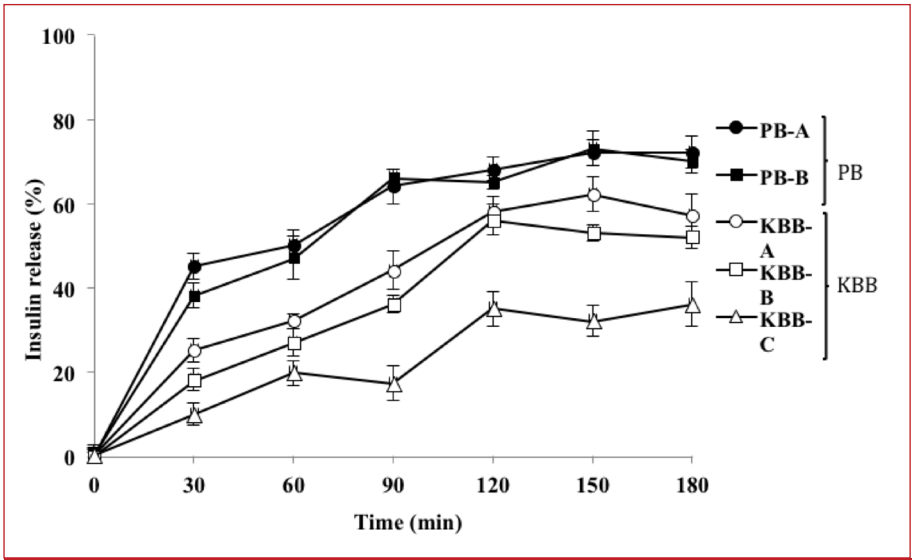

Figure 7: Insulin nanoparticle release profiles into phosphate buffers A and B (differences in electrolyte compositions), and Kreb's Bicarbonate buffers A (47:1 Na:Ca), B (10:1) and C (1:1).

it dissociates to monomeric form in neutral pH. These effects would not be as apparent with HPLC detection. Nevertheless, a comparison of figures 5 and 6 will show that rather similar amounts of insulin are detectable following 5h GI simulation, irrespective of the measurement method. Under the worst case conditions, 48% of the initial insulin remains detectable, in the absence of particle protection.

It is apparent from figure 4 that the most significant release of insulin from particles is triggered by the pH transition from the gastric to intestinal condition. In figure 7, release kinetics were examined at neutral pH but into different buffers commonly used for intestinal simulations. Phosphate buffer A (PB-A) is the most commonly used simulation buffer, but it is compared to Kreb's Bicarbonate buffers, which more closely represent the buffering condition of the intestine. The other differences between the buffers are in the electrolyte composition, and the Na:Ca ratio. It is apparent that phosphate buffer and high Na:Ca ratio strongly promotes insulin release due to displacement of Ca^{++} from the particle core, and the chelating properties of phosphate, while bicarbonate buffers and lower Na:Ca ratio reduces release by a half over 3h. As insulin assay was based on monoclonal antibody binding, it is assumed that fully intact insulin is being detected in the release medium. While bicarbonate better represents intestinal conditions, meal constituents including high/low sodium or calcium will impact on insulin release.

Future studies will be directed toward a better understanding of the nature of insulin quaternary structure and impact on insulin stability in simulated and real GI fluids, and the role of GI medium composition on the uptake of free and nanoparticulate insulin.

CONCLUSIONS

A nanoparticulate polymer complex containing human insulin is described providing the highest level of PA reported in the literature. Insulin instability in acid (gastric) and neutral (intestinal) medium including in presence of intestinal proteases, is neutralized when in nanoparticulate form. It is noteworthy that the extent of premature release of insulin and demonstrated stability in intestinal simulation, as well as the amount of retained particulate insulin are both consistent with the level of PA observed in vivo.

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Ronald Neufeld has degrees in Cell Biology (McGill) and Chemical/Biochemical Engineering (Western). After working in Engineering and Process Development in Industry, he served as Professor of Chemical Engineering at McGill University from 1980 to 1997, Associate Dean of Engineering at McGill from 1993 to 1997, Head of the Department of Chemical Engineering at Queen's University from 1997 to 2006, and as Professor at Queen's to the present.

He served under the Lady Davis Fellowship program at the Technion, Haifa, and at the Hebrew University Hadassah Medical School in Jerusalem, as Visiting Professor at ONIRIS in Nantes and the Ecole des Mines de Nantes, and at the Institute of Bioorganic Chemistry in Moscow. His research interests are in micro and nanoencapsulation and controlled delivery of biological and bioactive materials. He is the author/coauthor of 140 refereed publications.

TESTIMONY

In memory of Dr Horst Dautzenberg 1935-1998

Horst Dautzenberg achieved his diploma in Chemistry from the Technical University of Dresden in 1967. His research was devoted to studying reaction kinetics for the processing of celluloses at the Institute of Polymer Chemistry of the German Academy of Sciences in Teltow and received his master's degree at the Technical University in Magdeburg in 1970.

Besides studying reaction kinetics studies his interests were in practical applications of cellulose systems. In the beginning of the eighties his research was shifted and focused to the study of the preparation of microcapsules by polyelectrolyte complex formation of PDADMAC and cellulose sulfates. His research appeared in many scientific articles and patents.He was a very friendly person and established collaborations with many researchers.

He attended the first BRG conference in 1991 and was one of the most active member, especially organizing the international conference on bioencapsulation in 1996..

THESIS ABSTRACT

Formation of capsules by interfacial reaction with chitosan and characterizations

Carole PERIGNON

Supervisor Denis Poncelet (Oniris)
Gisèle Ongmayeb (Capsulae)
Date/place October 18th, 2013/ Nantes (France)
Affiliation Oniris

Abstract: The objective of this thesis is to develop an encapsulation method with the use of a green chemistry for encapsulation of sensitive actives and to extend applications especially in the food and cosmetic fields. Capsules with a diameter of 2,5 mm and an aqueous core have been formed by interfacial reaction between chitosan, a natural biopolymer, and a non-toxic reactive, dissolved in a vegetable oil. The process of capsule formation is a drop by drop system with a moderate agitation. The membrane of capsules is formed by grafting of the reactive on chitosan. The membrane thickness is homogeneous. The growth of the membrane would be progress by successive layers involving the same number of bonds between the reactants. The study of the mechanical resistance of the capsules has showed that the membranes have good flexibility. The mechanical resistance of capsules is related to the thickness of the membrane and it can be modified by varying chitosan concentration. The vitamin B1 release profile has showed good membrane permeability for low molecular weight molecules.

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ARTICLE

SYNTHESIS ROUTE FOR THE SELF-ASSEMBLY OF SUBMICRON-SIZED COLLOIDOSOMES WITH TAILORABLE NANOPORES

Bollhorst T., Grieb T., Rosenauer A., Fuller G., Maas M., and Rezwan K..

INTRODUCTION & OBJECTIVE

Colloidosomes are hollow capsules formed via self-assembly of colloid particles on emulsion droplets and were first termed by Dinsmore et al. (2002). This type of semi-permeable capsule possesses the potential to fabricate vesicles with tailored properties for the encapsulation and sustained release of active agents, such as drugs, flavors, or fragrances. Previous investigations have demonstrated colloidosome synthesis with fine-tuned properties. These include, increased mechanical stability (Ao 2011), adjustable permeability (Miguel 2011), synthesis of pH (Cayre 2012) and temperature (Lawrence 2007) responsive systems, and the integration of materials with magnetic, catalytic, or semiconducting characteristics. The stability and porosity of the capsules can be further enhanced by merging the particles on the shell by sintering (Yow 2009). However, the utilization of sintering techniques opposes various application limits for the encapsulation of active agents. Furthermore, a major property that has not been reported to date is the assembly of colloidosomes with diameters below 1 µm.

Accordingly, demonstrated colloidosome sizes and the limitations created by previously described stabilization procedures highlight the need for a novel synthesis route to fabricate inherently rigid colloidosomes of small size with intrinsic nanoporosity. In this study, we present a straightforward synthesis route for the fabrication of colloidosomes at mild pH and ambient conditions with diameters below 1 µm, featuring tailorable nanometer-sized pores.

Advanced CERAMICS

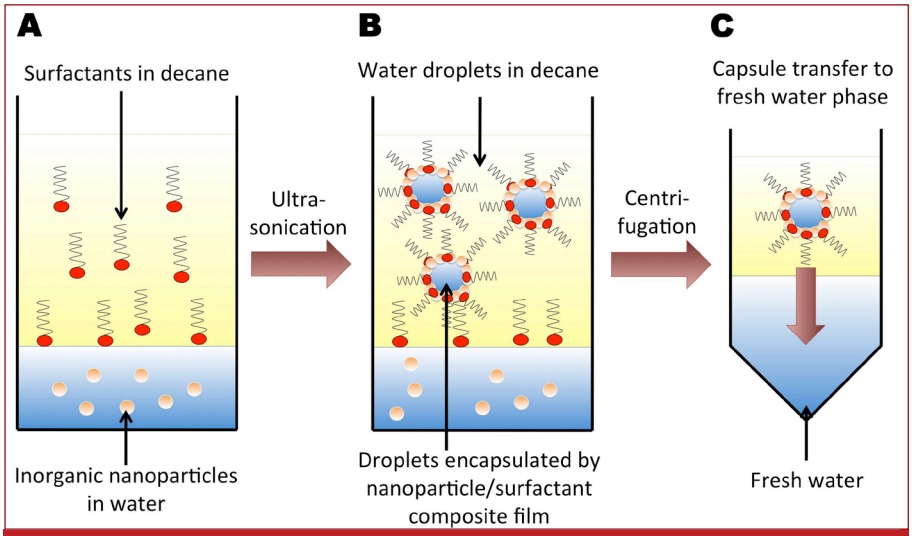

Figure 1: Submicron colloidosome preparation; (A) System before colloidosome generation; (B) Self-assembly of lipids and nanoparticles at the water-droplet oil interface; (C) Transfer of self-assembled colloidosomes to a fresh aqueous phase.

MATERIALS & METHODS

Our preparation method is primarily inspired by the standard procedure given by Dinsmore et al. (2002). Here, an emulsification step to prepare a water-in-oil emulsion is utilized to induce a self-assembly process of colloidosomes, which is followed by a centrifugation step to transfer the capsules from an organic into an aqueous phase (Figure 1).

Our mechanical stabilization of the colloidosomes is based on our previous study (Maas 2010), where we demonstrated the growth of silica nanoparticle thin-films at a planar water-oil (w-o) interface. We now transferred the method of a oil-soluble surfactant induced agglomeration of nanoparticles at a two-dimensional planar interface to a three-dimensional curved interface of water droplets, with the aim to obtain inherently rigid colloidosomes of submicron size. Akartuna et al. (2009) investigated a similar approach with water-soluble surfactants producing capsules with an oil core which exhibited diameters of a few micrometers.

In our work (Bollhorst 2013), submicron colloidosome synthesis is realized by combination of oil-soluble surfactants (lipids) and nanoparticles at the w-o interface, both carrying equal net charges. In our study, three colloid/lipid-combinations of equally charged nanoparticles and lipids were employed. Ludox TMA (Sigma Aldrich), colloidal silica particles that exhibit a negative surface charge, were used with stearic acid (Sigma Aldrich), which induces a negative charge at the w-o interface. While Alu C (Evonik) as well as Ludox CL (Sigma Aldrich) both feature a positive surface charge and were used with stearyl amine (Sigma Aldrich), that shows a positive charge at the w-o interface. Using colloid/lipid-combinations of opposite charges of the nanoparticles and lipids solely produce large agglomerates and were not applicable for colloidosome synthesis.

Table 1: Properties of sub-micron colloidosomes

Colloidosome type	Size distribution (nm)	Pore size (nm)	Surface structure
SiO_2	420 ± 160	7.9 ± 2	mostly hcp
Al_2O_3	230 ± 60	13.7 ± 9	unordered
Al_2O_3 - coated SiO_2	200 ± 140	4.2 ± 1	mostly hcp

RESULTS AND DISCUSSION

The DLS intensity size distributions obtained for the hydrodynamic diameters for the different colloidosomes types are listed in Table 1.

All three samples feature diameters in the range of about 100 nm up to a few hundred nanometers, confirming the submicron size of all three colloidosome types. Zeta-potential measurements indicated a high electrostatic stability of the colloidosomes in the aqueous phase after centrifugation.

The colloidosomes' shell and its pores potentially impact the diffusion path of an active agent and therefore the release from the capsules' cores. Hexagonal closed packing (hcp) provides the smallest possible pore size in colloidosomes, and can be ideally realized via the self-assembly of spherical nanoparticles of narrow size distribution. Figure 2 illustrates the surface structure of the different colloidosomes types. Pore sizes and the type of the surface packing created by the shell-forming nanoparticles are listed in Table 1.

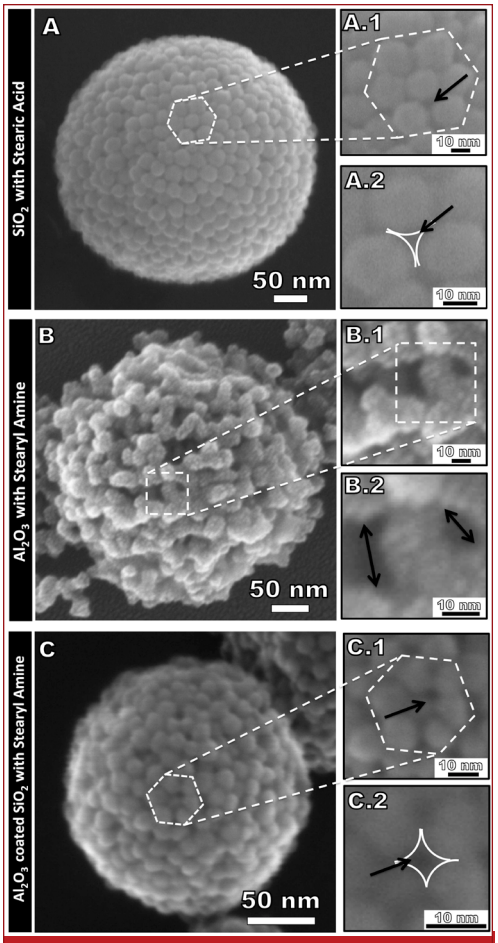

Figure 2. SEM micrographs illustrating the topological properties of the varying colloidosome types, including a detailed view of several nanopores. (A) SiO2 colloidosome (B) Al2O3 colloidosome (C) Al2O3 coated SiO2 colloidosome.

CONCLUSIONS

In summary, this study describes a straightforward method for the preparation of inherently rigid submicron-sized colloidosomes with tailorable nano-pores. In combination with a lipid that carries the same net charge at an oil-water interface as the colloidosome-forming particles in aqueous media, we were able to synthesize colloidosomes with positive and negative zeta potentials. The capsules are both stable in organic as well as in aqueous environments. The different colloidosome types are therefore potentially suitable for the encapsulation of positively or negatively charged biomolecules. By varying the sizes and shapes of the nanoparticles, we were able to tailor the pore diameters and pore size distributions on the surface of the capsules. Tailoring the size of the colloidosome nanopores potentially allows the controlled release of encapsulated active agents of different size, such as proteins or antibiotics.

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ACKNOWLEDGEMENT

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Tobias Bollhorst is currently a PhD student at the University of Bremen in Germany. His thesis focuses on submicrometer-sized capsules that are formed by inorganic nanoparticles and have great potential in the field of nanomedicine, specifically for targeted drug-delivery. Mr. Bollhorst spent one year in the USA where he studied Chemistry at the University of North Carolina Charlotte before pursuing his bachelor's degree in Materials Science at the Technical University of Berlin. He received his master's degree at the University of Bremen where he conducted an interdisciplinary master's thesis in the Electron Microscopy and Advanced Ceramics workgroups.

ARTICLE

ENCAPSULATION OF OIL IN CA-ALGINATE MICROCAPSULES BY INVERSE GELATION TECHNIQUE

Martins E., Renard D., Davy J., Marquis M. and Poncelet D.

INTRODUCTION AND OBJECTIVES

The oil encapsulation has been used in different industry sectors as agriculture, pharmaceuticals, foods, cosmetics and fragrance (Abang et al. 2012). This process is advantageous for various reasons, such as for conversion of liquid to solid form to facilitate handling, transportation or incorporation into other components, taste/smell masking, protection from evaporation or oxidation, and controlled-release applications.

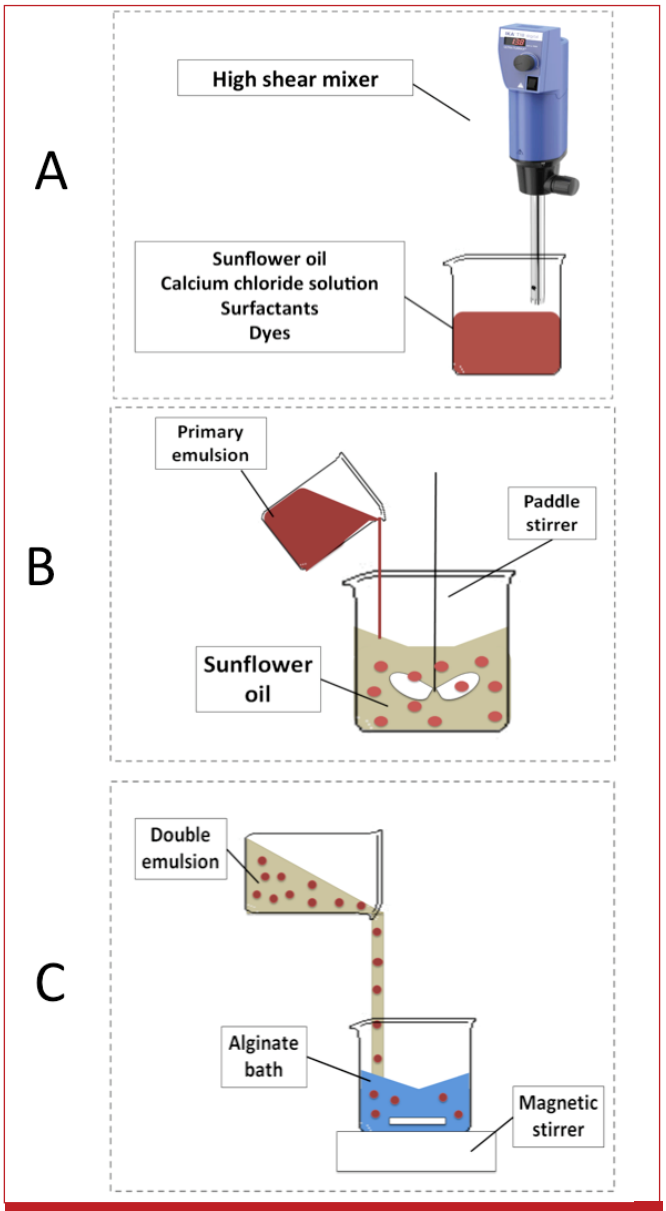

Figure 1. Diagram of the experimental set-up. Production of primary emulsion (A), double emulsion (B) and microcapsules (C).

A promising technique for oil encapsulation in Ca-alginate capsules by inverse gelation has been proposed by Abang et al. (2012). This method consists of emulsifying calcium chloride solution in oil then dropped into an alginate solution to produce aqueous-core calcium alginate capsules. This technique allows the production of capsules with diameters around 3.2 mm, though this size can be inappropriate for application in industrial products. As this size is a limiting factor for applications of these capsules in industry, the objective of this study is to propose a new process of oil encapsulation based on double emulsion in Ca-alginate microcapsules by the inverse gelation method.

MATERIALS AND METHODS

Sodium alginate powder Algogel 3001 was kindly donated by Cargill (France). Calcium chloride powder ($CaCl_2$. $2H_2O$) (Panreac Quimica Sau, Spain) and sunflower cooking oil (Associated Oil Packers, France) were used to prepare the emulsions. All other chemicals of analytical grade were obtained from Sigma Aldrich (France).

Preparation of alginate solution

Ten gram of alginate powder was dissolved in 1 L of demineralised water. A surfactant (Tween 85) at 0.5% v/v was then added to the alginate solution.

Preparation of primary emulsion and double emulsion

For the preparation of a primary emulsion, 100 mL of sunflower oil containing 0.5 mL of Tween 85, 0.5 mL of Span 85 and 0.01 g of Sudan red, were stirred using a high shear mixer (Ultra-Turrax T25, IKA, Germany) at 13 500 rpm during 30 s. Thirty mililiters of calcium chloride solution was then added slowly and a new shear mixing at 13 500 rpm for 3 min was performed (Figure 1A)

For the production of a double emulsion, 10 mL of primary emulsion was dispersed in 100 mL of sunflower oil using a paddle stirrer (Eurostar digital, IKA, Germany) at 500 rpm for 2 min (Figure 1B).)

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Experimental set-up

Hundred mililiters of double emulsion was added into 400 mL of alginate solution (Figure 1C). The alginate solution was stirred at 350–400 rpm with an 80 mm long wedge-shaped magnetic barrel. After 15 min of curing at ambient temperature (20 ± 2 °C), the wet capsules were washed with demineralised water to remove excess alginate and to prevent the capsules from sticking each other.

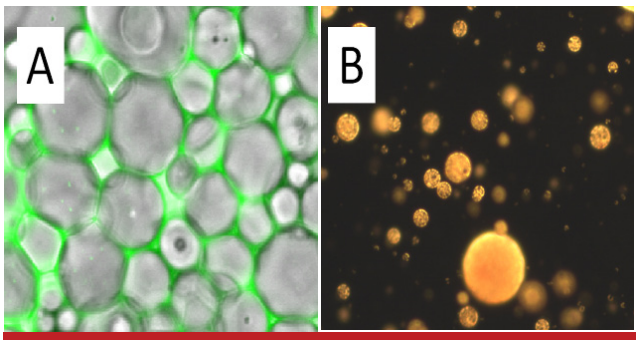

Figure 2. (A) Confocal microscopy of the primary emulsion (Green: Calcium chloride solution; Gray: Sunflower oil). (B) Double emulsion. Droplets of primary emulsion (yellow) dispersed in sunflower oil (black background).

Observation of emulsions and measurements of microcapsules diameters and thicknesses

A confocal laser-scanning microscope (Nikon, France) with a 4X objective was used to measure the diameters and the membrane thicknesses of the microcapsules. The same equipment was used for observation of the primary emulsion labeled with fluorescein (0.08 g/L).

RESULTS AND DISCUSSION

The first step for the preparation of microcapsules consisted in the production of a primary emulsion containing a source of calcium. This step was a crucial point once the little variations in the protocol could change the properties of primary emulsion.

Surprisingly, analyses by confocal microscopy revealed that the water-in-oil (W/O) primary emulsion became an oil-in-water (O/W) emulsion by catastrophic inversion (Figure 2A). Surfactants, with high and low hydrophilic-lipophilic balance (HBL), were used in this protocol in association with a high speed of shear, thus allowing the phase inversion of the primary emulsion (Scheer et al. 2013). This property allowed the dispersion of the primary emulsion in oil, ensuring the formation of a double emulsion (Figure 2B).

However, during the production of double emulsion, droplets of different sizes were formed, implying the formation of heterogeneous microcapsules in size.

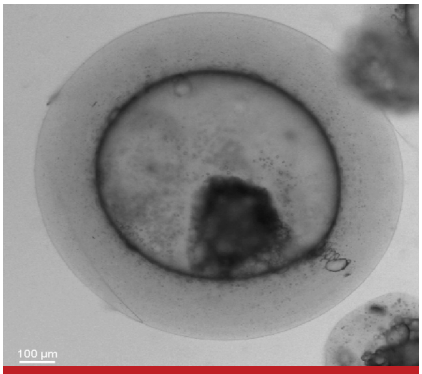

Figure 3. Ca-alginate microcapsules with oil core.

Table 1. Microcapsules characteristics

Parameters	Size (µm))
Microcapsules diameter	513 ± 135
Membrane thickness	121 ± 41
Core diameter	270 ± 94

For the production of microcapsules, the double emulsion was then added to the alginate bath. Thanks to the stirring of alginate bath, the droplets of primary emulsion of the double emulsion migrated outside the oil phase where they entered in contact with the alginate solution. During the curing time, the Ca^{2+} diffused through the primary emulsion, leading to the formation of an alginate membrane. At the end of the process (15 min), microcapsules with a Ca-alginate membrane and an oil core were formed (Figure 3).

This new process enabled the production of microcapsules with diameters of ~500 µm (Table 1). In addition, the membrane corresponded on average to 85 % of the volume of microcapsules. However, the membrane thickness was able to vary with the core diameter.

CONCLUSION

The new process developed allowed the production of Ca-alginate microcapsules by inverse gelation with a considerably higher membrane thickness compare to the classical process. Further studies on physical properties of microcapsules and controlled release of actives will be conducted in order to ensure for these capsules potential industrial application.

INRA SCIENCE & IMPACT

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ARTICLE

ENCAPSULATION OF STRUCTURED PHENOLIC LIPIDS BY COMPLEX COACERVATION METHOD

Aziz, S., Neufeld, R.J., and Kermasha, S

INTRODUCTION & OBJECTIVE

Due to its important biomass (Massrieh 2008), krill oil (KO) offers a new abundant source of ω-3-polyunsaturated fatty acids (ω-3-PUFAs), in particular eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3). The enzymatic transesterification of krill oil with the phenolic acid 3,4-dihydroxyphenylacetic acid (DHPA) resulted in the synthesis of novel bio-molecules, phenolic lipids (PLs), with enhanced anti-oxidative and solubility properties (Aziz et al. 2012). The entrapment of PLs within micron-sized particles could be an effective approach for their protection and delivery into the food system. As compared to other technologies, the complex coacervation, which involves the electrostatic attraction between two biopolymers of opposing ranges, offers several advantages including, higher payload, very low amount of surface oil and a relatively thick outer shell (Barrow et al. 2007). The objective of this study was to optimize the encapsulation of PLs, obtained by the transesterification of KO with DHPA, via complex coacervation.

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MATERIALS & METHODS

Materials

Kosher-certified Beef-hide gelatin (GE) was obtained from Vyse Gelatin Co. Gum arabic (GA) was purchased from ACP Chemicals Inc. High-Potency krill oil, extracted from Euphausia superba, was generously obtained from Enzymotec Ltd. Commercial immobilized lipase, Novozym 435, from Candida antartica with an activity of 10,000 propyl laurate units per g solid enzyme, was purchased from Novozymes A/S. The 3,4-dihydroxyphenylacetic acid (DHPA) was purchased from Sigma Chemical Co. Sodium hydroxide, ammonium hydroxide, ethanol, glacial acetic acid and organic solvents of high-performance liquid chromatography (HPLC) grade were purchased from Fisher Scientific.

Microencapsulation

The coacervated particles were produced according to a modification of the method of Liu et al. . The microencapsulation was carried out in a double-jacketed reactor, linked to a circulator-bath. Stock solutions of gelatin (1%, w/v) were prepared and their pH was adjusted, from 5.8 up to 8.5, by the addition of 0.25 N sodium hydroxide. A defined amount of the synthetized PLs was emulsified into GE, using a homogenizer as well as a sonicator ultrasonic liquid processor at a ratio of core material to wall (RCW) of 1.25:1. The GA (1%, w/v) was then added to the GE-stabilized emulsion and the mixture was stirred for an additional 5 min, followed by an acidification to pH 4.0 by the dropwise addition of 10% (v/v) acetic acid to induce the complex coacervation. The heating unit of the circulator-bath was then turned off and the mixture was allowed to slowly cool from 45±3°C to room temperature over time under constant mechanical stirring at 400 rpm. The particle suspension was then slowly cooled, with continuous stirring, to 10°C using an ice-bath. The coacervate rich phase was then recovered and stored at 4°C for further analyses.

Determination of encapsulation efficiency & morphology of microcapsules

The total and surface oils were determined according to a modification of the method of Liu et al. and Drusch et al. (2006), respectively. The encapsulation efficiency (% EE) was calculated following the equation: ((total oil - surface oil)/total oil x100). The morphology of the microcapsules was observed on a hemacytometer, using a stereomicroscope equipped with a camera. The diameters of the microcapsules were measured using ImageJ Analysis Software.

Statistical analysis

One way Anova statistical analysis was used to determine the difference among several groups, followed by the Holm-Sidak test for pairwise comparisons.

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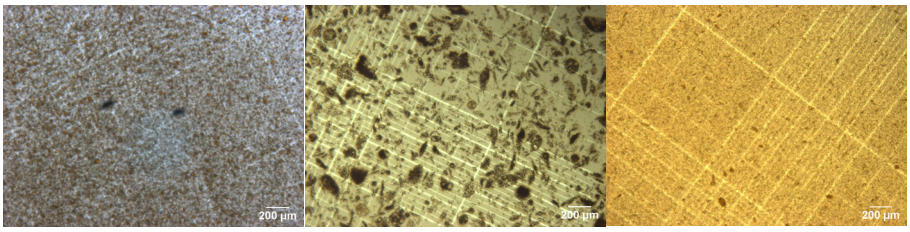
RESULTS & DISCUSSION

Effects of the phenolic acid and phenolic lipids, present in the esterified KO, and the emulsification device on the emulsion: The experimental findings showed (Figure 1a) that a stable homogenous emulsion was obtained, when KO was emulsified into GE, at a homogenisation rate of 20,500 rpm. However, when the esterified KO (EKO) was emulsified into GE, under the same conditions, small particles of irregular shapes were formed at the emulsification stage (Figure 1b).



a b c

Figure 1: Emulsions of gelatin-krill oil (a) & gelatin-phenolic lipids (b & c), as observed by a stereomicroscope, using as the emulsification device, the homogenizer (a & b) and the ultrasonic liquid processor (c).

ARTICLE

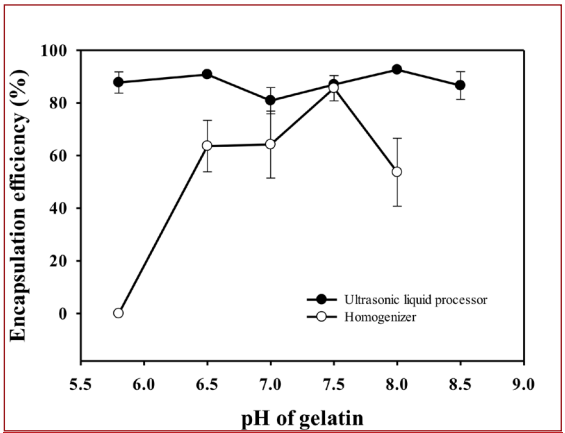


Figure 2: Effects of pH of gelatin & emulsification device on the encapsulation efficiency of phenolic lipids.

These findings could be due to the fact that the homogenizer created shearing by applying a tangential force to the mixture which could have favored the interactions between the PLs and the phenolic acids in the oil with the gelatin (Burden 2008). These results are in agreement with those of Zhang et al. (2010), who confirmed that the phenolic compounds do react with the amino groups in GE leading hence to the formation of crosslinks. In order to improve the stability of the emulsion, the effect of using the ultrasonic liquid processor was investigated. Figure 1c shows that the emulsion, obtained with the ultrasonic liquid processor was homogenous, stable and free of particles. These findings could be due to the fact that the ultrasonication may have resulted in an emulsion by applying shock waves using differential pressure, which prevented the formation of particles between the GE and the EKO (Burden 2008).

Effects of pH of gelatin and emulsification devices on the EE and the size of the capsules: Using the homogenizer at pH 5.8, which corresponds to that of the beef GE, the formed capsules were susceptible to breakage, which made the determination of EE difficult (Figure 2). In addition, the formation of these capsules, at the emulsification stage, had an influence on the size of the microcapsules. Zhang et al. (2010) reported that the cross-linking of GE with phenolic acids was obtained under alkaline conditions.

Using two emulsification devices, the effects of the pH of GE on the EE and the size of the microcapsules were investigated. The experimental findings indicated that using the homogenizer and varying the pH of GE from 5.8 to 8.0, there was a statistically significant difference ($P < 0.05$) in the EE of PLs (Figure 2), but statistically insignificant difference ($P > 0.05$) in the size of the PLs microcapsules (Table 1). On the other hand, using ultrasound and varying the pH of GE from 5.8 to 8.5, there were no statistically significant differences in the EE and in the size ($P > 0.05$) of the PLs microcapsules (Figure 2, Table 1).

a Standard deviation; b Not determined.

Table 1: Effects of pH of gelatin & emulsification device on the size of the capsules.

pH	Homogenizer	Ultrasonic liquid Processor
	Size of capsules (μm)	
5,8	626 ±127[a]	608 ±121
6,5	708 ±16	457 ±22
7,0	705 ±72	477 ±47
7,5	594 ±10	417 ±3
8,0	677 ±12	408 ±6
8,5	ND[b]	515 ±12

CONCLUSION

Overall, the ultrasonic liquid processor was found to be a more appropriate device for the emulsification of the EKO in GE, as compared to the homogenizer. The EE, size and stability of the EKO microcapsules were dependent on the nature of the emulsion. Unlike the homogenizer, using the ultrasound, the pH did not have any significant effect on the EE of microcapsules, with an EE ranging from 80 to 92%.

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ARTICLE

SUPERCRITICAL FLUID EXTRACTION OF EMULSIONS FOR THE PRODUCTION OF VITAMIN E NANOENCAPSULATES

Prieto C. and Calvo L.

INTRODUCTION & OBJECTIVE

SFEE (Supercritical Fluid Extraction of Emulsions) is a new particle formation technology (Chattopadhyay 2006). It uses supercritical CO_2 (sc CO_2) to rapidly extract the organic phase of an emulsion, in which a bioactive compound has been previously dissolved. By eliminating the solvent, the solute precipitates, which generates a suspension of particles of that compound in water. The produced particles have controlled size and morphology, due to the use of the emulsion, which acts as a template, and to the fast kinetics of the sc CO_2 extraction, which avoids particle agglomeration. This method has been mainly used for the entrapment of a compound within a secondary material which protects and stabilizes the core from a variety of physical and chemical factors. Up to now, little work was done, and most of it was related to pharmaceutical compounds encapsulated in PLGA (poly(lactic-co-glycolic acid)). However, this method is a promising technique to encapsulate nutraceuticals for the food industry.

The aim of this work was the application of this technology for the production of nanostructures of vitamin E (vit. E), which is considered one of the most important antioxidants for human nutrition. It has high sensitivity to light, heat and oxygen and it is not soluble in water, what can be avoided by its encapsulation. Furthermore, the encapsulation of a liquid is a very interesting application for the future production of 'liquids into powders'.

MATERIALS & METHODS

Materials

α-tocopherol (≥95%), polycaprolactone (PCL) (MW of 10,000), Tween 80, acetone (≥99,5% (GC)), ethanol (absolute) were all from Sigma Aldrich and used as received. Millipore water was used throughout the study.

Sample preparation

The emulsions were formed accordingly to the following procedure: a) addition of the adequate quantity of oil, in which the vit. E (0.51 wt. %) and the coating polymer, PCL (0.63 wt. %) were previously dissolved at 40ºC, b) addition of the adequate quantity of surfactant, c) mechanical shaking during 1 min on the vortex, d) addition of the adequate quantity of water, e) vigorous mechanical shaking for 5 min on the vortex in order to guarantee a homogeneous dispersion, f) control of temperature at 40ºC, by placing the tube within a thermostatic water bath.

Study of the phase behaviour

Twenty compositions in weight were chosen to cover the whole ternary phase diagram, in order to differentiate between emulsion and microemulsion and select the best composition to be then subjected to SFEE.

Formation of the nanoparticles by SFEE

The supercritical extraction apparatus consisted in a 100 ml cylindrical stainless steel vessel in which 50 g of emulsion were placed for each experiment. Liquid cooled CO_2 was delivered using a high pressure membrane pump (Milroyal D, Milton Roy) and was introduced into the extraction vessel at a constant flow rate of 1g·min^{-1}. The temperature in the extractor was regulated by a heating jacket and read inside the vessel by a thermocouple type K within ± 1ºC. The pressure in the separator was regulated by a backpressure valve (BPR) and read in a Bourbon manometer within ± 3 bar. A mass flow meter (AlicatScientific, M-10SLPM-D) was used to read the total amount of CO_2 employed. A scheme of the equipment is shown in Fig. 1

Product characterization

To determine the encapsulation efficiency, the suspension of nanoparticles was centrifuged at 15,000 rpm (Digicen 21). The pellet was washed with ethanol and the concentration of vit. E in the supernatant was analysed by UV-vis spectrophotometry (MRC UV1800).

The loading capacity was calculated as the rate between the encapsulated vit. E and the amount of polymer employed.

A sample of the nanoparticles was studied by SEM (JEOL JSM 6335F) and TEM (JEOL JEM 1010) to analyse their morphology.

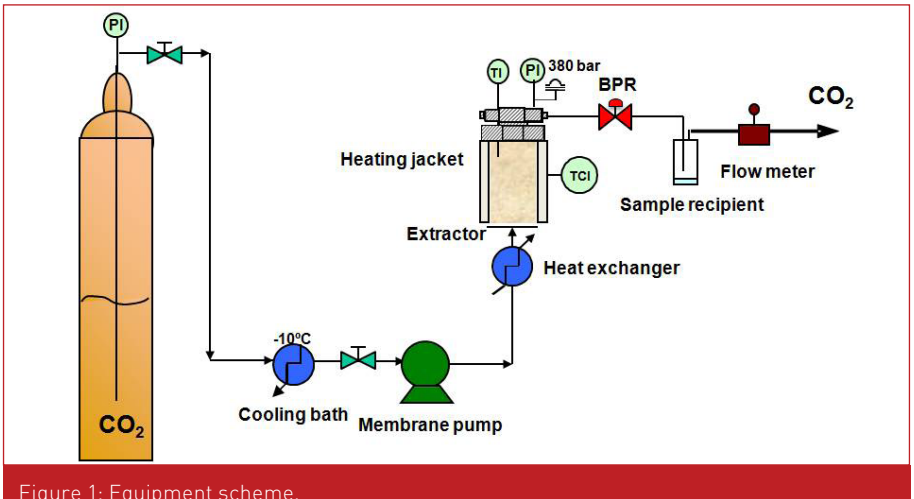

Figure 1: Equipment scheme.

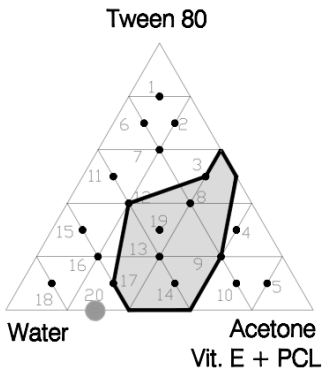

Figure 2: Phase map of the system Tween 80 / acetone + vit. E + PCL / water.

RESULTS & DISCUSSION

Determination of the phase behaviour

The phase behaviour of this system was obtained by visual observation of each sample, and plotted as a pseudoternary phase diagram shown in Fig. 2.

The system shows three behaviours represented with different shading: area without shading or microemulsion; area with a light grey shading where PCL precipitated, corresponding presumably to the bicontinuous structure; and finally, sample 20 which corresponds to the emulsion region. This sample, 71.6% of water, 28.3% of acetone and 0.07% of Tween 80 in weight, was then selected to create the nanoparticles.

Formation of the nanoparticles by SFEE

The formation of the nanoparticles of vit. E by SFEE is based on the precipitation of the polymer once the organic solvent is extracted from the emulsion by the supercritical CO_2. The result of this operation is a suspension of the encapsulated nanoparticles in water, which could be recovered by centrifugation and/or by evaporation at room temperature.

The operation conditions were selected to get a complete extraction of acetone without extracting the vit. E, according to solubility data of acetone (Katayama 1975) and vit. E (Chrastil 1982). The temperature and pressure selected were 40ºC and 80 bar, respectively because at these conditions, sc CO_2 and acetone were completely miscible and the solubility of vit. E very low.

Other parameters that can affect acetone extraction are the extractor design, the CO_2 flow rate. With the optimal configuration (L/D=9) and with a flow of 0.67 g·min^{-1}, 24 min of operation time were needed to guarantee the complete extraction of the acetone.

Product characterization

The nanoparticles suspension was centrifuged, washed with ethanol, and then analysed by UV-vis spectrophotometry. The encapsulation efficiency was calculated assuming that none of the vit. E was extracted by sc CO_2, as the difference between the initial amount and the one dissolved in the ethanol. Thus, the encapsulation efficiency was around 80%. This result was similar to that obtained by Khayata (2012) using the nanoprecipitation method for the same system. Thus, the loading capacity was around 64%.

Regarding to nanoparticles morphology, images obtained by SEM and TEM (See Fig. 3) showed that the particles were spherical and with size around 300 nm. No particle agglomeration was observed.

CONCLUSIONS

The SFEE technology was successfully applied to the production of vit. E nanoencapsulates. High encapsulation efficiency and high loading capacity were obtained. Particle size was of the order of magnitude of the starting emulsion droplet. Morphology was also adequate. Furthermore, despite being a high pressure technology, moderate pressure was used, so the compression costs could be easily overcome by the added value of the product. We are working now on the optimization of the emulsion characteristics regarding to the concentration of the Tween 80 and acetone to reduce the raw materials costs, and to the ratio between vit. E and PCL to increase the coverage ratio. Optimization of the extractor design will also reduce the operation time.

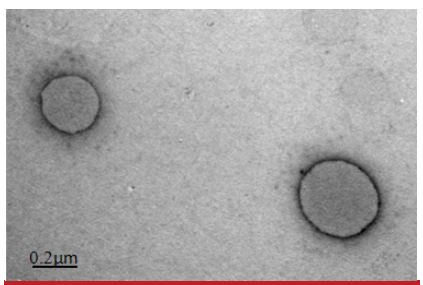

Figure 3: TEM of vit. E loaded nanocapsules.

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ARTICLE

NOVEL ALGINATE BASED AMPHIPHILIC DRUG-CONJUGATED GRAFT COPOLYMERS FOR CONTROLLED CO-DELIVERY OF ANTI-TUMOR AGENTS: CURRENT PROGRESS IN POLYMER SYNTHESIS

Kapishon V, Cunningham M, Neufeld R, Whitney R and Champagne P.

INTRODUCTION

Amphiphilic polymers have been widely used in encapsulation and delivery of hydrophobic drugs due to their ability to self-assemble in aqueous solution into micelles, polymersomes, vesicles, nanotubes, etc. An amphiphilic polymer has distinctive water soluble and water insoluble regions in a molecule. Alginate, a naturally found electrolyte polysaccharide, was chosen to be a hydrophilic block due to its biocompatibility. Less water soluble or hydrophobic polymers were synthesized using single electron transfer living radical polymerization (SET-LRP), a relatively recent polymerization technique with greater control over molecular weight and polydispersity compared to traditional free radical polymerization. Other polysaccharides have been previously modified with polymers via LRP methods (Voepel 2011). Because alginate, in its natural form, is a high molecular weight polymer, it was partially degraded to produce low molecular weight fragments (Xiaoxia 2010) and modified to be soluble in organic solvents for further reactions (Pawar 2011).

We are currently working on two different synthetic approaches to make alginate based amphiphilic polymers. First one is a 'graft from' strategy, where the hydrophilic polymer is grown directly from alginate. Second is a 'graft onto' approach, where a polymer is synthesized first and then clicked onto alginate.

Recent advances in drug delivery show that self-assembled vesicles with drugs chemically conjugated to the polymer produce more controlled and sustained drug release (Wenchuan 2013, Miller 2013, Harrison 2013). Therefore, we decided to chemically link two anti-tumor drugs, Oseltamivir Phosphate (OP) and Gemcitabine to the polymers for controlled co-delivery of chemotherapy.

Successful production of alginate based amphiphilic polymers capable of encapsulating the desired materials is a greener alternative to petroleum based polymers and could find its use in other applicable fields such as industrial controlled release systems, latexes and others.

MATERIALS AND METHODS

Alginate Preparation

High molecular weight (HMW) alginate was depolymerized in the presence of H2O2 and ascorbic acid according to Xiaoxia (2010).

Acidified LMW alginate was neutralized with tetrabutyl ammonium (TBA) hydroxide to give alginate-TBA salt which was confirmed by H-NMR.

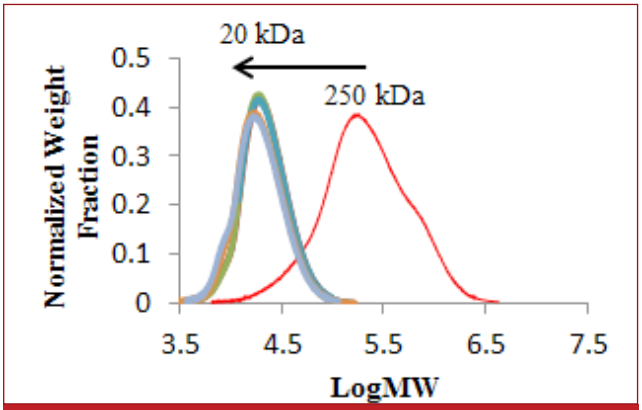

Figure 1 : aq-GPC of original and degraded alginatesodium alginate

'Graft From' Synthesis

Alginate-TBA was reacted with α-bromoisobutyric acid (α-BrIBA) (SET initiator) in DMF/TBAF solution via steglich esterification in presence of DCC/DMAP to give Alg-Br macroinitiator. OP was then conjugated to alginate-Br via EDAC aided coupling in water to give OP-Alg-Br. Polyethylene glycol methyl ether methacrylate (PEGMEMA), methyl methacrylate (MMA) or MMA/t-butyl acrylate was then grafted from OP-Alg-Br via SET-LRP. All steps were confirmed by H-NMR and aq-GPC.

'Graft Onto' Synthesis

HO-PEG-NH_2 (1.2 kDa) was reacted with $(BOC)_2O$ in water to produce HO-PEG-NH-BOC, which was esterified with α-BrIBA to give α-BrIB-O-PEG-NH-BOC initiator. MMA or MMA/t-butyl acrylate was first co-polymerized from a standard ATRP initiator (EBIB) via SET-LRP to establish appropriate reaction time and conditions. Same polymerization was then repeated with amine protected initiator to give polymer-NH-BOC which was then deprotected in acid to produce reactive amine specie (polymer-NH_2).

OP was coupled to LMW alginate as previously described to give OP-Alg-COOH, which was later reacted with polymer-NH_2 via EDAC aided coupling.

RESULTS & DISCUSSION

Reduction in MW was confirmed by dynamic light scattering (DLS) and aqueous gel permeation chromatography (aq-GPC) (Figure 1). After de-

ARTICLE

gradation and solubility modification LMW alginate was functionalized with SET initiator with a degree of substitution of 7%. H-NMR of macroinitiator is shown in Figure 2. Grafting from synthesis was then accomplished by SET-LRP from Alg-Br. As an example, Figure 3 shows H-NMR of the final product, Alg-pMMA co-polymer. At high concentration, the product self-assembled into micelles (average size 70nm by DLS) which could be observed by TEM. Formation of stable micelles during SET-LRP reactions provided a proof of successful synthesis of amphiphilic material capable of self-assembly in aqueous environment. Similar grafting from route is currently being followed but with OP covalently conjugated to alginate. A small amount of t-BA is now co-polymerized into the hydrophobic polymer to provide a number of reactive sites for coupling with gemcitabine in the future.

This will result in co-delivery of the two drugs from two different regions of the micelle in a sustained fashion.

Grafting onto approach first involved preparation of hydrophobic polymer prior to linking to alginate. Figure 4 shows H-NMR of p(MMA-co-tBA) with Mw of 7-10kDa. We have finalized appropriate reaction time and conditions to give a hydrophobic fragment with a desired MW (~10kDa). In case of p(MMA-co-tBA) copolymers, as in grafting from approach, tBA is later transformed into acrylic acid which is capable of peptide bonding with Gemcitabine.

Finally, drug conjugated micelles will be tested for sustained drug release in-vitro and anti-tumor efficiency in-vivo.

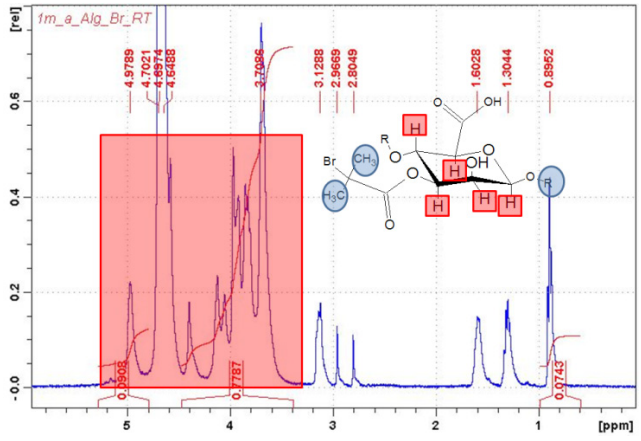

Figure 2 : H-NMR of alginate-Br macroinitiator

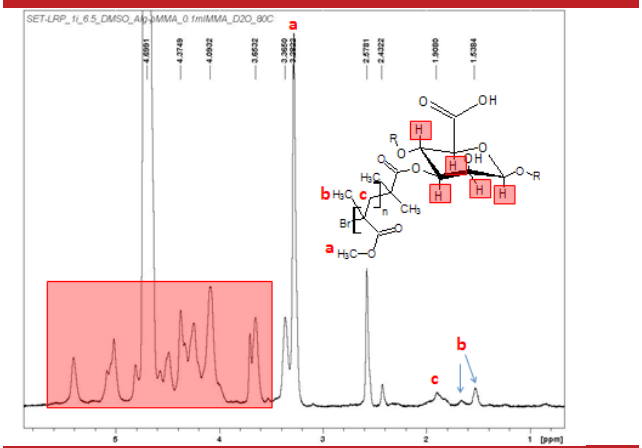

Figure 3 : H-NMR of alginate-pMMA

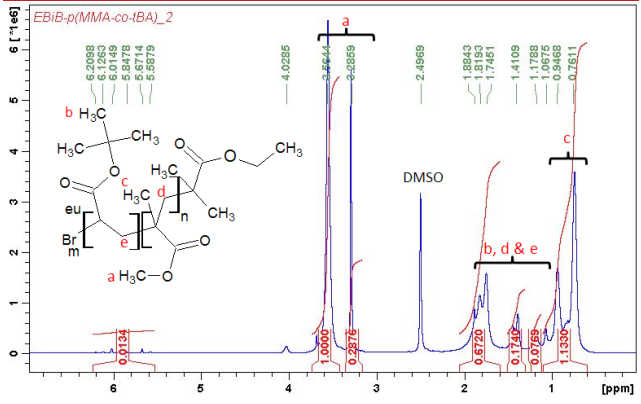

Figure 4 : H-NMR of EIB-p(MMA-co-tBA)-Br

CONCLUSION

Alginate based amphiphilic polymers with chemically linked drugs show a potential in the area of drug release and biomaterial engineering.

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ARTICLE

ROLE OF DANGER SIGNALS FROM THE ENVELOPED CELLS IN IMMUNE RESPONSES AGAINST MICROENCAPSULATED HUMAN ISLETS

Paredes-Juarez, G.A., de Haan, B.J. and de Vos, P.

INTRODUCTION

Transplantation of islets is proposed as a therapy to cure diabetes type 1. However, the outcomes for using this treatment are variable and it still requires the use of immunosuppressant (de Vos et al., 2006). Encapsulation of pancreatic islets is a solution for this issue (de Vos et al., 2007). This technique is employed to protect the cells from the immune response of the host. Graft survival however is limited (Stokes et al., 2013) which can be attributed to factors related to the capsules and the enveloped islets.

The role of the latter, i.e. the islets, has received not more than minor attention. Immune responses against encapsulated islets can occur after binding of islet-derived molecules to pattern recognition receptors (PRRs). These PRRs are able to bind to proinflammatory islet-derived molecules, such as damage-associated molecular patterns (DAMPs), that can be released when islets in the capsules are under stress. Toll-like receptors (TLRs) are the most commonly known PRRs. We hypothesize that the islets release proinflammatory islet-derived components that can elicit inflammatory responses via TLRs.

The present study is intended to investigate the role of components derived from the islets as triggering factors of the immune response against islets in the first weeks after implantation.

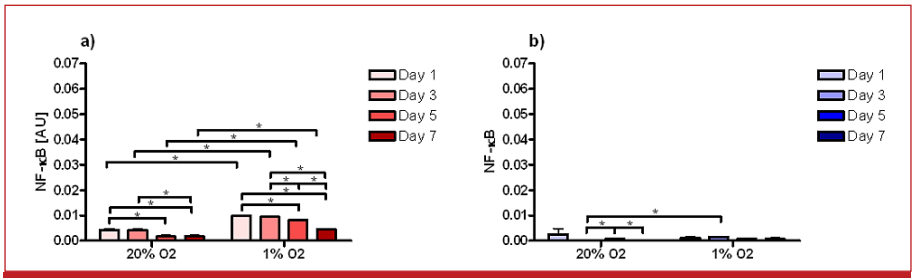

Figure 2: NF-kB activation dependency on MyD88(-) cell-line of control (a) and nutrient deprivation model (b) at 20 and 1% O2 of islets. Values are presented as mean±SEM (n=4); $p<0.05$(*)

MATERIALS & METHODS

Cell stimulation

Two cell-lines were stimulated using supernatant from human islets cultured in starving conditions at 20% and 1% O2 for detection of proinflammatory components. The islets were analyzed using live/dead staining for fluorescent confocal microscopy. Samples were collected during days 1, 3, 5, and 7. We applied the following cell-lines: THP1-MD2-CD14 (MyD88(+)), a human cell-line carrying all TLRs with a reporter plasmid under control of the NF-κB, expressing a secreted embryonic alkaline phosphatase (SEAP) gene that can be measured. The THP1-defMyD (MyD88(-)) cell-line has the same construction as MyD88(+) but it has a non-functional MyD88 coupling protein and it can be used to prove activation via TLRs in a MyD88 dependent manner.

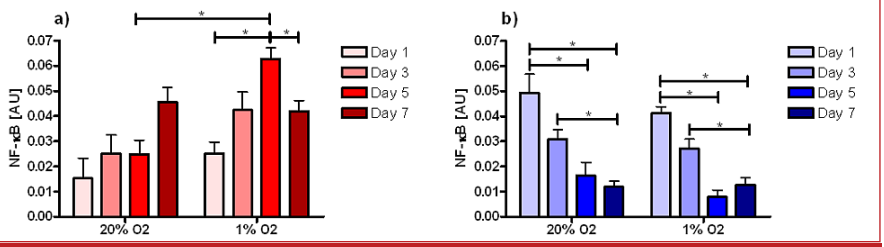

Figure 1: Effects of control (a) and nutrient deprivation model (b) at 20 and 1% O2 of islets in activation of NF-kB in MyD88(+) cell-line. Values are presented as mean±SEM (n=4); $p<0.05$(*)

Confocal analysis

LIVE/DEAD Viability/Citotoxicity Kit from InvitroGen was used. Collected islets were incubated with Calcein AM (1 mM) and Ethidium Bromide (EB) (2 mM) at room temperature avoiding light. The islets were washed with KRH. Fluorescent confocal microscopy was measured at an emission wavelength of 517 nm (Calcein AM) and 617 nm (EB).

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RESULTS & DISCUSSION

As encapsulated islets are not revascularized after transplantation they face low oxygen condition and nutrient deprivation after transplantation. We study the effect of this on immune activation. We mimicked in vitro conditions at which human islets are exposed to low oxygen tensions and to lower concentrations of nutrients than in the vascularized condition. This was done by culturing islets in control medium (20% oxygen and 10% FCS) and comparing their activation of NF-κB on the monocyte line THP-1 with that of islets cultured on hypoxic conditions (1% oxygen) and nutrient deprivation (0% FCS).

As shown in Figure 1, islets produce under all in vitro conditions compo-

nents that activate NF-κB in THP-1 cells, including the normal control conditions of 20% oxygen and 10% FCS. This increases during prolonged periods of culture illustrating progressive damage of the islets.

The production of proinflammatory molecules increased significantly under hypoxic conditions (Figure 1a). This increase in activation was twofold higher. This was different when nutrient deprivation was applied. In this case we found a strong release of proinflammatory islet-derived molecules in the first days but decreased fast thereafter. This can be explained by pronounced cell-death due to nutrient deprivation in the first days as will be explained below.

We next questioned whether the immune response was Toll-like receptor (TLR) dependent or also dependent on other pattern recognition receptors. We therefore compared the responses of THP-1 cells containing functional and non-functional MyD88, i.e. the main signaling molecule for TLR. As shown in Figure 2, the responses are two to fourfold lower in the THP1 MyD88(-) implying that the islet derived factors are mainly signaling via TLRs but that also via other families of pattern recognition receptors. With nutrient deprivation the effects were even more extreme. There was an approximate tenfold decrease in activation. The effects of other PRRs are minimal but still there.

Confocal analysis was used to assess the viability of the islets under different conditions using live/dead staining with Calcein AM (green, live) and EB (red, dead). As shown in Figure 3 control islets do not present an obvious change in the viability of its cells from day 1 to 7. They remain viable as illustrated by the predominant green staining. Under hypoxic conditions more cells are in the active process of dying (red stain) or are necrotic which results in transparent ghost cells. This is even worse and associated with more cells in the process of dying when hypoxia and nutrient deprivation are combined.

CONCLUSIONS

Human islets exposed to conditions they face after transplantation in encapsulated cells result in release of islet-derived factors that stimulate immune cells in a NF-κB dependent fashion. This NF-κB activation is mainly TLR dependent. Our data suggest that inhibition of TLRs should be applied in, at least, the first week after transplantation when encapsulated cells face a novel microenvironment with hypoxia and nutrient deprivation.

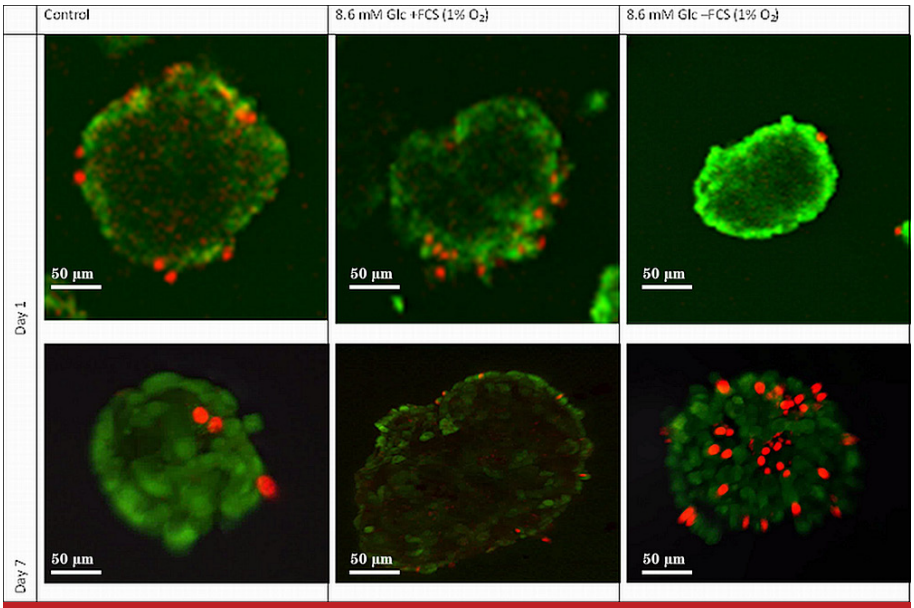

Figure 3. Microscope confocal images of islets stained with Calcein AM (green, live cells) and Ethidium Bromide (red, dead cells)

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In Mexico I accomplished a Bachelor in Biology in 2006, and a Master in Biotechnology. In 2008, both at the University of the Americas, Puebla (UDLAP). I did my master thesis in the area of human physiology, specially the alterations caused by diabetes, and I became interested in different aspects of this disease. After that, I started working as a teacher at the UDLAP at the Department of Sciences. Now I'm a PhD student at the UMCG at the Department of Pathology and Medical Biology, my research line focus on the development or improvement of new materials for immunoisolation and transplantation of Langerhans islets in patients with diabetes type 1 avoiding, both, immunosuppression and graft rejection.

ARTICLE

INTRACELLULARLY TRIGGERED BIOPOLYMER BASED POLYELECTROLYTE NANOCAPSULES FOR TARGETED DELIVERY OF ANTICANCER DRUGS

Pulakkat S., Balaji S., Rangarajan A. and Raichur A.M.

INTRODUCTION & OBJECTIVE

Targeted delivery of anticancer therapeutics can minimize most of the drug-originated systemic toxic effects. Micro- and nanometer sized polymeric capsules have emerged as potential candidates for applications in the fields of drug delivery, sensing, catalysis and microreactors (Tong 2012). Herein, we report a polyelectrolyte nanocapsule system capable of targeting CD44 receptors on breast cancer cells and releasing its cargo at intracellular lysosomal pH. Hollow nanocapsules were fabricated by Layer by Layer (LbL) technique using hyaluronic acid (HA) and protamine (PR). HA, besides being the structural component of the capsule, also acts as a targeting ligand.

The size, morphology and surface characteristics of the nanocapsules were characterized followed by loading of an anticancer drug, doxorubicin (dox). The cellular uptake of the nanocapsules was evaluated by fluorescence-activated cell sorter (FACS) and confocal microscopy. MTT assay revealed that the drug loaded nanocapsules exhibited superior antitumor effect over the free drug.

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MATERIALS & METHODS

Preparation and Characterization of nanocapsules

Hyaluronic acid, protamine, Dulbecco's modified eagle medium and doxorubicin hydrochloride were purchased from Sigma Aldrich.

LbL technique involves sequential deposition of oppositely charged polyelectrolytes onto a sacrificial template. Briefly, silica nanoparticles were incubated alternatively with PR and HA solutions and after deposition of 6 layers, the template was removed to obtain hollow capsules. The surface charge of the coated particles was measured after every adsorption step. Morphological characterization of the nanocapsules was done by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).

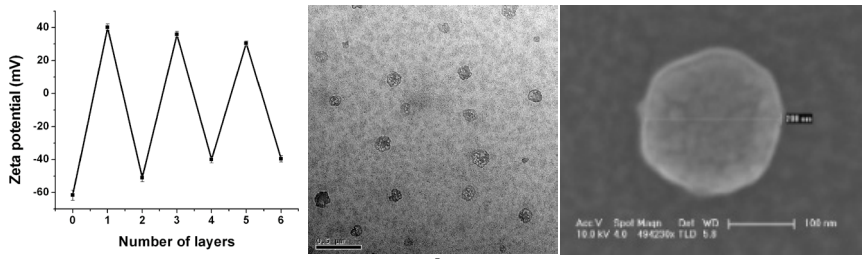

Fig 1: (a) Zeta potential (b) TEM and (c) SEM analysis of hollow nanocapsules

Drug loading and release studies

The hollow nanocapsules were incubated overnight with dox at pH 3. The sample pH was then raised to 5 followed by several washings. The sample was centrifuged and the amount of drug in the supernatant was estimated by measuring the absorbance at 490 nm using a spectrophotometer. The dox loaded nanocapsules (HA-PR-dox) were later suspended in pH 4 acetate buffer to monitor the drug release, and the cumulative release percentage was estimated.

Cellular uptake and cytotoxicity studies

Breast cancer cell lines MDAMB 435S (CD44+) and BT 474 (CD44-) were used for in vitro studies. About $1x10^5$ cells were grown with DMEM supplemented with 10% FBS at 37°C incubator and 5% CO_2 supply.

The binding efficiency of nanocapsules to CD44 receptor was evaluated by incubating free dox and HA-PR-dox with MDAMB 435S and BT 474 cells for 1 hour followed by PBS wash and trypsinization. To study the effect of HA pretreatment, the cells were incubated with HA solution before adding free dox and nanocapsules. Both samples were then subjected to FACS analysis. Confocal images were obtained after incubating MDAMB 435s cells with 1 µM of free dox and HA-PR-dox for 12 hours.

The in vitro toxicity of the HA-PR-dox nanocapsules was assessed using MTT assay over a range of concentrations. The IC50 values for free dox and HA-PR-dox were also determined.

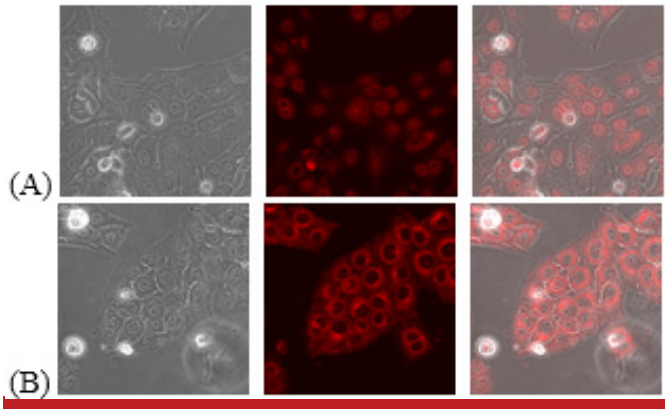

Fig 2: Confocal images of MDAMB 435S cells treated with (A) free dox and (B) HA-PR-dox

ARTICLE

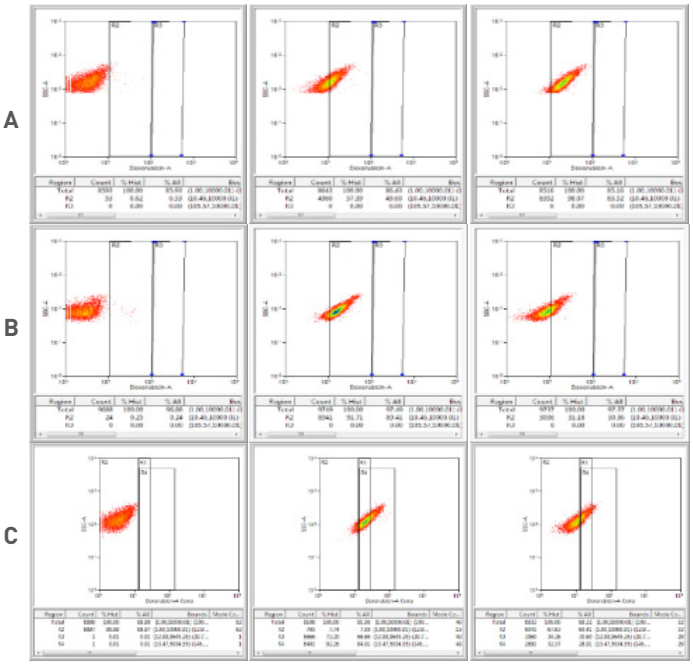

Fig 3: FACS analysis (i) unstained cells, (ii) free dox and (iii) HA-PR-dox (A) MDAMB 435S cells, (B) BT 474 cells, (C) Cells treated with HA

RESULTS & DISCUSSION

Preparation and characterization of nanocapsules

The LbL assembly was carried out at an assembly pH of 5 considering the pKa and pI values of HA and PR respectively. At this pH, both polyelectrolytes are highly charged resulting in strong electrostatic attraction between the multilayers (Szarpak 2010). The zeta potential measurements yielded a saw tooth graph indicating the charge reversal due to alternate deposition of positively and negatively charged PR & HA respectively (Fig 1A). The coated particles were then treated with 1 M HF to yield hollow capsules. The SEM and TEM analysis showed nanocapsules in the size range of 150-200nm (Fig 1B, 1C). The removal of the template was confirmed using EDX analysis (data not shown here).

Drug loading and release studies

Dox was loaded into the nanocapsules at an acidic pH and 64% loading was achieved. At pH 3, which is close to the pka value of HA, the electrostatic attraction between the multilayers decreases leading to increased permeability and hence diffusion of drug into the capsules. Drug release studies at pH 4 showed a sustained release of dox over a period of 48 hours, the cumulative release being 42% (data not shown here).

Cellular uptake studies

The cellular uptake of nanocapsules were studied in MDAMB 435S (CD44+) and BT474 (CD44-) cell lines. FACS studies showed that HA-PR-dox nanocapsules, with HA as the final layer, are internalised more rapidly in MDAMB 435S cells. It was also observed that more amount of encapsulated dox was found inside the cells than free dox (Fig 3A, 3B). When the cells were pre-treated with HA solution, the uptake was found to be less (Fig 3C). This indicates that the CD44 receptors were saturated with free HA molecules, thereby confirming that nanocapsules are internalised by receptor mediated endocytosis. CLSM images also revealed an increased delivery of drug inside the cells using HA-PR-dox capsules (Fig 2).

Cytotoxicity assay

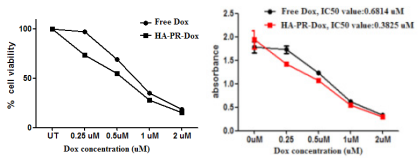

Fig 4: Comparison of (A) cytotoxicity and (B) IC50 value of free dox and HA-PR-dox nanocapsules

MTT assay in MDAMB 435S revealed that HA-PR-dox was more cytotoxic than free dox (Fig 4A). The IC50 values obtained for free dox (0.6814 µM) and encapsulated dox (0.3825 µM) also confirmed that HA-PR-dox was more efficient in killing cancer cells (Fig 4B). The acidic pH and enzymes in the lysosomes trigger the sustained release of dox, thereby enhancing the antitumor effect of HA-PR-dox

CONCLUSION

In summary, 6 layered HA/PR nanocapsules were prepared and characterized. The pH permeability of capsules enabled efficient loading and release of dox. The final layer HA, being a ligand for CD44 receptors, facilitated targeted delivery of HA-PR-dox in CD44 over expressing breast cancer cells. The enhanced uptake of nanocapsules and the sustained release of the encapsulated dox ensured superior anticancer effect than free drug.

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Sreeranjini. P completed her Bachelors in Biomedical Engineering from Calicut University and Masters in Nanomedical Sciences from Amrita Vishwavidyapeethom, India. Currently she is working on her PhD thesis under the supervision of Ashok M Raichur in the Biomaterials laboratory at the Department of Materials Engineering, Indian Institute of Science, Bangalore, India. Her research focuses on the development of the micro-and nanosized polymer capsules and nanoparticles towards controlled and targeted drug delivery applications. She is experienced at designing and developing polymeric systems to suit diverse requirements of biological applications and their characterization.

ARTICLE

THE PROJECT ATTRACT: PROTECTION OF CROPS FROM SOIL-BORNE INSECT PESTS WITH A NOVEL ATTRACT-AND-KILL STRATEGY

Humbert, P., Vemmer, M., Beitzen-Heineke, W., Kleeberg, H., Vidal, S., Patel, A.

INTRODUCTION AND OBJECTIVE

The project ATTRACT targets the development of a novel attract-and-kill strategy for the protection of crops from soil-borne insect pests.

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Larvae of herbivorous insects (e.g. wireworms, western corn rootworm, black vine weevil) cause severe losses in many crops (potato, maize, strawberry). A control of these pests with soil insecticides is severely restricted or has recently been abandoned. The project ATTRACT aims at developing innovative attract-and-kill formulations which can be produced on technical scale and can then be used as novel control strategies against soil-borne insect pests in conventional as well as organic farming systems. By attracting larvae to the beads containing a kill compound (Fig. 1) insecticide applications or other control strategies can be replaced, the amount of insecticides can be minimized and the environment and health of farmers and consumers can be protected.

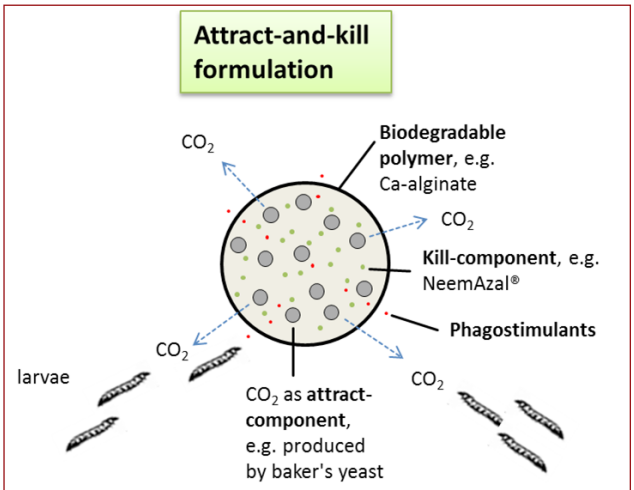

Figure 1: Attract-and-kill formulation. The bead consists of a biodegradable polymer and includes a CO_2-producing component (e.g. baker's yeast) and an environmentally friendly insecticidal compound, e.g. NeemAzal®. Released CO_2 and phagostimulants attract the larvaes (not true to scale) towards the attract-and-kill formulation.

Baker's yeast has proved to be a suitable CO_2-releasing source (Vemmer 2011) and thus can be used as attract-component. The kill-component is represented by environmentally friendly insecticidal compounds, e.g. NeemAzal®, an Azadirachtin-enriched plant extract of the Neem tree (*Azadirachta indica* A. Juss.), resp. a plant extract of the Quassia-tree (*Quassia amara*) named Quassia-Extrakt-MD (both TRIFOLIO-M, Germany). Neem products are used in traditional Indian medicine since many centuries and nowadays some of these products arouse much interest because of their insecticidal effects. Also the botanic Quassia-tree promises some interesting pest-control properties (Kleeberg 2006).

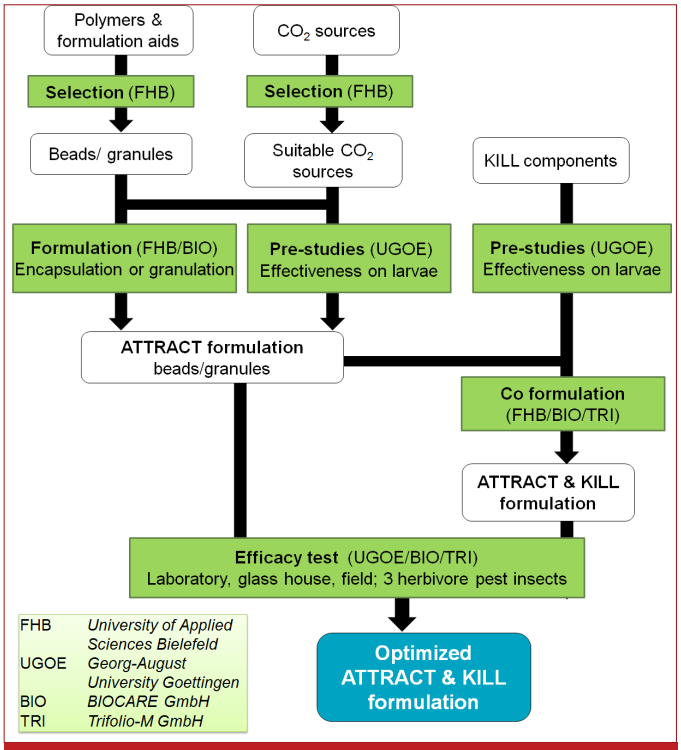

Figure 2: Flow diagram for the project ATTRACT with distribution of responsibilities.

Organization and scheduling of the project ATTRACT

Within the project ATTRACT four partners are cooperating at an interdisciplinary level. Beside the University of Applied Science Bielefeld, the Georg-August-University Göttingen and the SME's BIOCARE GmbH (Einbeck, Germany) and TRIFOLIO-M GmbH (Lahnau, Germany) are involved in the project (Fig. 2).

MATERIALS & METHODS

Encapsulation of *Saccharomyces cerevisiae* and Co-encapsulation with NeemAzal® or Quassia-Extrakt-MD

Commercial baker's yeast mixture was encapsulated in moist Ca-alginate beads to serve as CO_2 source. The baker's yeast mixture was also co-

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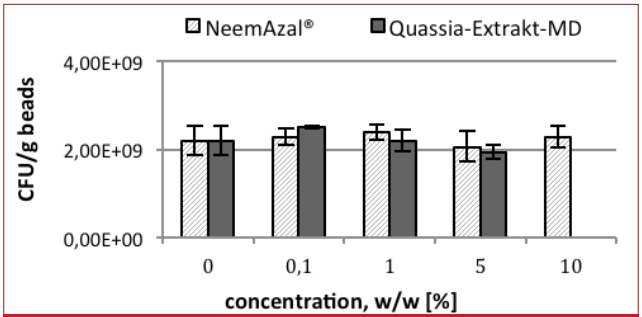

Figure 3: CFU per g beads for baker's yeast co-encapsulated with NeemAzal® and Quassia-Extrakt-MD after one week of storage. Initial CFU: 2,19 x 10^9/g beads was nearly maintained for all tested concentrations of plant extracts (SD always < 4,00 x 10^8).

encapsulated with the plant extracts NeemAzal® and Quassia-extract-MD (both TRIFOLIO-M, Germany).

Examination of the compatibility of *Saccharomyces cerevisiae* and NeemAzal®, resp. Quassia-Extrakt-MD Co-encapsulation and dissolving of beads

After distinct times of storage at 25 °C alginate beads containing 20 % baker's yeast and different concentrations of NeemAzal® or Quassia-Extrakt-MD were dissolved in a solution consisting of 0.05 M Na_2CO_3, 0.02 M citric acid, pH 6.8. The dissolved beads were serially diluted in 0.9 % NaCl solution and defined volumes were plated on YPD-Agar. Following this, CFU was determined.

Filter disc assay:

Filter discs (Ø 9 mm) were dipped into solutions of different concentrations of NeemAzal® or Quassia-Extrakt-MD in 50/50 (v/v) EtOH/H_2O until complete saturation. After evaporation of the solvent the filter discs were placed on YPD agar plates, which had been inoculated with baker's yeast before. The plates were incubated for two days at 25 °C. A Dimetomorph containing solution was used as positive control and EtOH/H_2O served as negative control.

RESULTS & DISCUSSION

Examination of the compatibility of *Saccharomyces cerevisiae* with NeemAzal® or Quassia-Extrakt-MD

Because the attract-component and the environmentally friendly insecticidal compounds as kill-components should be encapsulated together within the attract-and-kill formulation, the tolerance of baker's yeast for NeemAzal® or Quassia-Extrakt-MD had to be examined. After four weeks of storage baker's yeast co-encapsulated with high concentrations of NeemAzal® or Quassia-Extrakt-MD still displayed a high degree of viability (Fig. 3).

The filter disc assay (Fig. 4) confirmed these results. For none of the tested plant extract concentrations a larger inhibition zone than for the negative control could be observed, indicating that NeemAzal® and Quassia-Extrakt-MD both had no negative effect on the growth of baker's yeast.

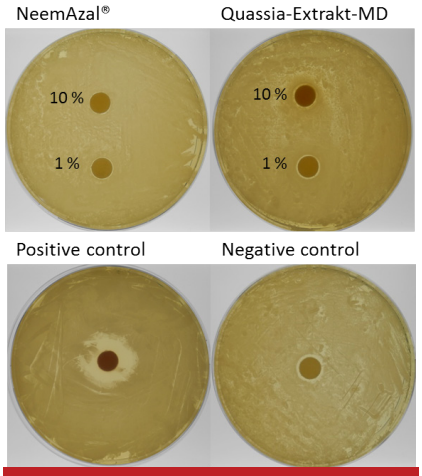

Figure 4: Filter disc assay for NeemAzal® and Quassia-Extrakt-MD concerning the growth of *Saccharomyces cerevisiae*.

CONCLUSIONS & OUTLOOK

In the project ATTRACT novel formulations (beads, granules) based on CO_2 emitting sources and environmentally friendly insecticidal compounds such as neem and quassin will be developed and tested under practical conditions in order to lure larvae away from plant roots. The first data indicate compatibility of baker's yeast and NeemAzal®, resp. Quassia-Extrakt-MD, which allows further development of attract-and-kill formulations based on these compounds. These formulations will be optimized by screening for different CO_2 sources, polymers, beads and granules. Further developments include the incorporation of phagostimulants and additives and the use of different coatings. Subsequently the formulations will be tested in efficacy tests in lab, greenhouse and field experiments.

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Pascal Humbert has both obtained his B.A.Sc. (2010) and M.A.Sc. (2013) in Molecular Biotechnology from the Bielefeld University. During his Master's thesis he worked on the genetic engineering of the amino acid producer Corynebacterium glutamicum with the aim to achieve an enhanced production of histidine. The work was supervised by Jörn Kalinowski. Currently he is working on his phD thesis at the Bielefeld University of Applied Sciences under the supervision of Anant Patel in cooperation with Karsten Niehaus (Bielefeld University). His research focuses on co-encapsulation methods for cells and insecticidal plant extracts for agricultural applications.

ARTICLE

ENZYME BIOENCAPSULATION USING MAGNETIC Fe_3O_4-CHITOSAN

Costa-Silva, T.A., Marques, P.S., Souza, C.R.F., Said, S., Oliveira, W.P.

INTRODUCTION

Application of enzymes can be achieved more economically and efficiently by immobilization to enhance its activity, selectivity, and operation stability. However, the immobilized derivatives are difficult to separate from the reactive medium, except though the use of high-speed centrifugation. Enzymes immobilized in the presence of Fe_3O_4 nanoparticles have the advantage of being easily and effectively recovered by application of magnetic fields. The aim of this work was to investigate the potential of bioencapsulation method for *C. kikuchii* lipase immobilization using magnetic Fe_3O_4-chitosan microparticles. Two different techniques were evaluated: entrapment by cross-linking with sodium tripolyphosphate (TPP) and spray drying.

MATERIALS & METHODS

Lipase Production

The *Cercospora kikuchii* lipase production was carried out according to Costa-Silva et al. (2011) and used for immobilization process.

Preparation of magnetic chitosan and enzyme immobilization

Fe_3O_4 nanoparticles were prepared by coprecipitaion method with a ferrous complex in presence of NH_4OH (Xie et al. 2009). Firstly, $FeCl_2.4H_2O$ and $FeCl_3.6H_2O$ [Fe^{2+}:Fe^{3+}=1:2] were dissolved in about 100 ml dionized water at a final concentration of 0.3 M iron ions. Next, this iron solution source was added drop-wise into NH4OH under strong agitation and subsequently the solution was heated at a constant temperature of 80 °C for 30 min and then filtered and washed with distilled water and ethanol. Finally, the resultant precipitates were dried in oven at 102 °C and add to the chitosan acetate solution. The suspension cross-linking technique was used for the preparation of magnetic chitosan microparticles and enzyme immobilization.

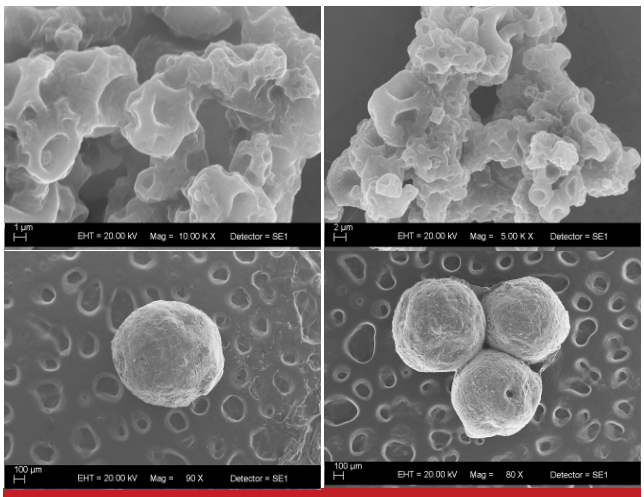

Figure 2: Scanning electron photomicrographs of microparticles: A) Powder produced by spray dryer B) Beads produced by cross-linking.

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In this specific procedure, a 4% chitosan solution was prepared using a 5% aqueous acetic acid solution containing Fe_3O_4 dry magnetic nanoparticles (Fe_3O_4 content/chitosan ratio: 1/4) and 20 ml of lipase solution (1.2 g of lipase). The contents were stirred gently to ensure complete homogenization. This solution was then extruded dropwise through a peristaltic pump into a beaker containing 1000 ml of 0.136 mM sodium tripolyphosphate ($Na_5P_3O_{10}$) solution, which was prepared in 0.05 mM Tris–HCl (pH 8.0). The formed beads were recuperated by simple filtration and dried by oven (35 °C) for 24 hours – Figure 1A. The magnetic chitosan acetate solution containing the enzyme was also dried by spray drying. Drying was conducted in a bench-top spray dryer (model SD-05, Lab-Plant, Huddersfield, U.K.), with a concurrent flow regime. The feed flow rate of atomizing air was set at 17.0 L/min at a pressure of 147.1 kPa. The flow rate of the drying air was maintained constant at 60 m^3/h. The drying conditions were performed according to Costa-Silva et al. (2011). The Figure 1B shows the powder produced after spray drying.

Figure 1. Lipase immobilized onto chitosan microparticles. A) Beads produced after cross-linking using sodium tripolyphosphate B) Powder produced after spray drying

Enzymatic activity

Lipase activity assay was performed using ρ-nitrophenyl palmitate (p-NPP) as substrate according to Mayordomo et al. (2000). One unit (U) of lipase activity was the amount of enzyme that released 1 µmol of p-nitrophenol/min under the aforementioned conditions.

The activity retention (REA %) was calculated following the equation (1):

$$REA\ (\%) = 100\ x \frac{\text{Immobilized enzyme activity } \left(\frac{U}{mg}\right)}{\text{Soluble enzyme activity } \left(\frac{U}{mg}\right)}$$

Residual enzymatic activity determination for immobilized lipase after 5 batch of reaction (five cycles) was carried out. The immobilized derivative was recovered by centrifugation and washed with buffer (sodium phosphate buffer 50 mM, pH 6.5) for the next reuse.

RESULTS & DISCUSSION

Table 1 shows the effect of immobilization conditions on outlet drying gas temperature (T_{go}), process yield, moisture content, and residual lipase activity (REA), of the immobilized derivatives.

Table 1: Dryer performance and product properties after bioencapsulation process

	Bioencapsulation Method	
	Spray drying	**Cross-linking (TTP)**
T_{go} (°C)	69.0±0.5	-
T_{in} (°C)	55.0±0.9	-
RE (%)	53.5±1.4	-
REA (%)	85.9±1.2	75.6±1.6
aw (-)	0.3±0.03	0.2±0.02
Moisture (%)	5.8±0.5	3.8±0.8
Five cycles	25.9±1.1	52.8±1.4

T_{go}: outlet drying gas temperature; T_{in}: bed temperature; RE: process yield; aw: water activity; REA: residual lipase activity.

The immobilization of lipase onto chitosan acetate powder obtained by spray drying process was more suitable for lipase activity retention than the cross-linking method. However, when it is considered the enzyme activity after five batches the use of sodium tripolyphosphate as a cross-linking agent for the beads production was the best immobilization condition. It was found that the immobilized derivative prepared by cross-linking method retained an activity of about 52.8% using p-NPP as substrate, after five reuses. In general, low values of moisture content (and water activity as well) are excellent for product stability. The moisture content in obtained immobilized derivatives varied between 3.8 and 5.8 % (Table 1). The water activity is another factor that affects the enzyme stability. The water activities of immobilized derivatives are in the range 0.2-0.3. These low values are important because the dehydration could provide an acceptable protein shelf life, ease storage and transport and protect the biological activity of these molecules. The yield of immobilized lipase powder production by spray dryer was 53.5 %.

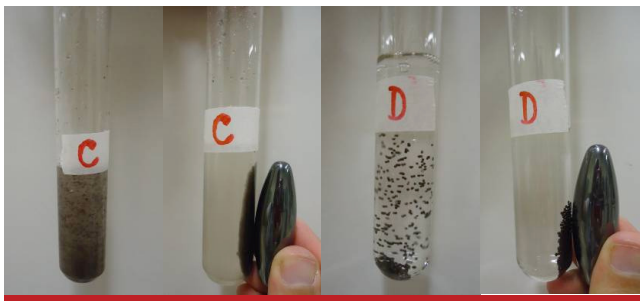

Figure 3: Dispersion and magnetic effect of immobilized derivatives

The outer morphologies of the immobilized derivatives are illustrated in Figure 2. The particles obtained by spray dryer did not show a defined format and the particle surface appeared grooved. For beads obtained by cross-linking method, the particle surface showed a rounded and well-defined shape. Figure 3 shows that the immobilized derivatives dispersed well in water, and aggregated within 10 s when a permanent magnet was nearby. The magnetic property will assist in the process of separation of the enzyme of the reaction medium and open new perspectives for enzymes utilization.

CONCLUSIONS

This work describes a successful method of magnetic chitosan microparticles production suitable for Cercospora kikuchii lipases immobilization. The bioencapsulation of lipase onto these particles showed little loss of enzyme activity, and the stability for the reuse cycles. Due to the good biocompatibility of chitosan, these particles may be used in magnetic-field assisted drug delivery, enzyme or cell immobilization and many other industrial applications.

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ACKNOWLEDGMENTS

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Mr Costa E. Silva has experience in microbiology with emphasis on: Fermentation Technologies, Industrial Enzymology (Production, Purification, Characterization and Immobilization of Enzymes) and Antimicrobial Activity using secondary metabolites produced by fungi. Furthermore, possesses experience in the operation and optimization of pharmaceutical processes developed in drying equipment like as spray dryers and spouted bed. Finally, we use bioencapsulation process to immobilize enzymes to enhance its activity, selectivity, and operation stability..

ARTICLE

ENCAPSULATION OF THYME AND OREGANO CO_2 EXTRACTS IN CA-ALGINATE HYDROGELS FOR ANTIMICROBIAL APPLICATIONS

Vemmer M., Fredrich E., Brune I., Tauch A., Patel A.

INTRODUCTION & OBJECTIVE

Because of the increasing resistance to drugs by some pathogenic microorganisms there is a renewed interest in the use of natural products like plant extracts as antimicrobial agents. The focus of this research is to develop formulation methods that stabilize the active substances, control their release and provide an appropriate handling.

For our encapsulation research we chose *Corynebacterium jeikeium* as a model for a multidrug-resistant microorganism. *C. jeikeium* is a "lipophilic" and multidrug-resistant bacterial species of the human skin flora. It is the most frequently recovered medically significant corynebacterial species at intensive care facilities and has been recognized with increasing frequency as a serious nosocomial pathogen.

MATERIALS & METHODS

Plant compounds

CO_2 plant extracts were kindly provided by FLAVEX Naturextrakte GmbH, Germany. Individual components of plant extracts were purchased with appropriate purity from usual chemical companies.

Preparation of emulsions

Oil-in-water emulsions of various lipophilic CO_2 plant extracts or lipophilic individual components of plant extracts were prepared as follows. The components were heated up to 60 °C to lower the viscosity. First, one part lipophilic substance was mixed with one part emulsifier (vortex mixer for µL scale; ULTRA-TURRAX® for mL scale). Then, water was added to a resulting concentration of at most 250 mg/mL for both. The concentrated emulsions can be diluted with water as required.

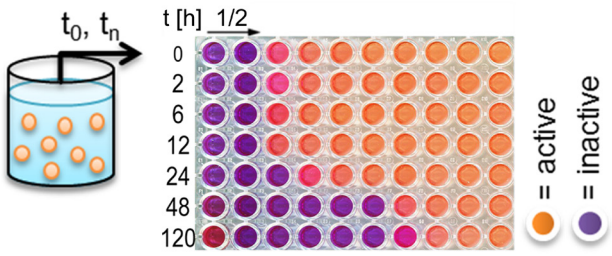

Figure 1: Release of thyme se extract in dH_2O and quantification using a MIC assay

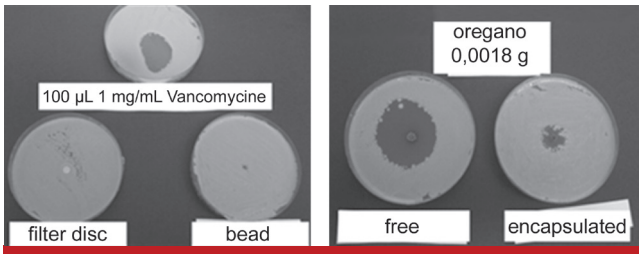

Figure 2: Agar diffusion test with a positive (vancomycine) and a negative control (filter disc and bead without active ingredients) and with oregano extract.

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Encapsulation of CO_2 plant extracts

Lipophilic CO_2 plant extracts were encapsulated with a concentration of 250 mg/mL in Ca-alginate hydrogel beads without any emulsifier or with 10 mg/mL Tween® 80.

Cultivation of *C. jeikeium*

C. jeikeium K411 originally isolated from the human axilla (Kerry-Williams and Noble, 1984) was grown at 37 °C in BYT complex medium, consisting of 37 g/L brain-heart broth, 10 g/L yeast extract, 1% (v/v) Tween® 80 (Tauch et al., 2004) and optionally 15 g/L select agar.

Agar diffusion test

In a first screening it was tested if different free or encapsulated CO_2 plant extracts show any antibacterial potential against *C. jeikeium* in agar diffusion tests.

MIC assay

Minimum inhibitory concentrations for emulsions (MIC_E) of different CO_2 plant extracts and for lipophilic individual components of plant extracts were determined with a broth dilution method. Therefore, a *C. jeikeium* culture (exponential growth phase) was diluted to a concentration of $2*10^4$ cells/mL (OD_{600} = 0.0002). The broth was incubated at 37 °C on a microtitre plate in wells containing progressively lower concentrations of the testing substance (100 µL total volume per well). After 24 h 30 µL 0.01 % Resazurin, a visual indicator, which changes its colour from blue to pink in the presence of viable cells, was added and after another 24 h, the MIC, which corresponds to the concentration in the blue well with the highest dilution, was determined.

Determination of release kinetics

To investigate the release kinetics of the active ingredients encapsulated plant extracts (with 10 mg/mL Tween® 80) were placed into dH_2O and incubated at 37 °C. The accumulation of active ingredients was tested by making use of the MIC test (Fig. 1). At certain points in time, samples were taken, mixed with Tween® 80 to a concentration of 10 % (v/v) to stabilize the emulsion and diluted, if necessary, with water to obtain sample concentrations of 90 %, 80 % and 60 % (v/v). The samples were serially diluted on a microtitre plate and handled according to the MIC assay. The accumulation is expressed via the dilution which is needed to obtain a

minimum inhibitory concentration for emulsions of release samples (MIC_R). This so called accumulation factor can be defined as the theoretically concentration (c_t) divided by MIC_R.

RESULTS & DISCUSSION

Agar diffusion tests

For oregano leaf extract, thyme leaf extract and sage leaf extract agar diffusion tests with 1% (v/v) Tween® 80 showed considerable inhibitory effects for the pure extracts on filter discs and the corresponding encapsulated CO_2 extracts (Fig. 2). After three days the pure extracts showed greater inhibition zones than the corresponding beads. That is probably due to a slowed release of the active substances from the beads

MICs

Different emulsifiers were tested and Tween® 80 has proved to be appropriate for the stabilization of emulsions. Affected by Tween® 80, the MICE of thyme and oregano CO_2 extracts is significantly lower than that of the principle components (p-cymene, carvacrol, thymol), even in combination (Tab. 1).

Table 1: MIC_Es for emulsions of different CO_2 plant extracts and for lipophilic individual components affected by Tween® 80

	MIC [µg/mL]
Oregano to CO_2 extract	50
Thyme se CO_2 extract	39
Thyme to CO_2 extract	62,5
p-Cymene	>2500
Carvacrol	1000
Thymol	1000
Thymol + Carvacrol (1:1)	1000
Thymol + p-Cymene (1:1)	>1250
Carvacrol + p-Cymene (1:1)	>1250
Thymol + p-Cymene + Carvacrol (1:1:1)	>1250
Rifampicine (control, without Tween® 80)	12,5

Broth dilution methods are widely used to determine MICs but they cannot so easily be used for hydrophobic compounds. The assays are often fitted by using agents like emulsifiers which on the one hand stabilize the test medium but on the other hand incorporate the active substances within the micelles whereby the antimicrobial activity may be influenced. Tween® 80 probably causes further changes in the physicochemical properties of the test system, because it is used as substrate by *C. jeikeium*.

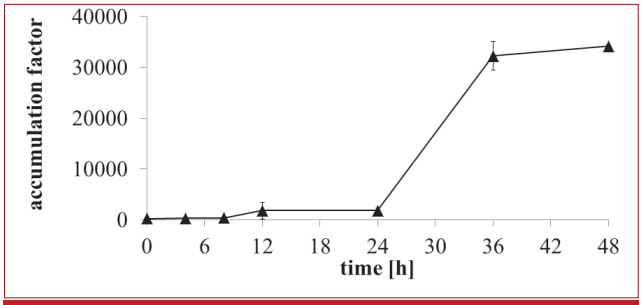

Figure 3: Release of the active compounds of encapsulated thyme se CO_2-extract

Release kinetics

The active substances of e.g. thyme se CO_2-extract accumulate in the medium (Fig 3).

Because the MIC test system is probably influenced by the concentration of Tween® 80 MIC_E and MIC_R should not be equated why the accumulation factor was introduced. Nevertheless and despite the limitations mentioned above the presented method is sufficient to describe release kinetics. Moreover, the test system is a suitable method to display the release of all active substances collectively in contrast to methods that detect only individual components. As we still can't link the high activity of CO_2 plant extracts (thyme, oregano) to one or more principle components (p-cymene, carvacrol, thymol) it is more suitable to observe the efficacy in total than the concentration of single substances.

CONCLUSIONS

Hydrogel encapsulation is principally suitable for slow release of some CO_2 plant extracts. The presented test system for the detection of MICs and for the characterization of release kinetics will be optimized and still more fitted to release experiments with hydrophobic compounds. Further investigations will deal with characterization of encapsulation materials, bead morphology and emulsifiers as well as their influence on the release kinetics.

Moreover, other individual components of thyme and oregano CO_2 extracts will be tested solitary and in combination.

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Marina Vemmer has both obtained her B.A.Sc. (2008) and M.A.Sc. (2010) in Molecular Biotechnology from the Bielefeld University. During her Master's thesis she worked in a classical biotechnological field on the cultivation of cells for the production of technical enzymes under the supervision of Erwin Flaschel. Currently she is working on her phD thesis at the Bielefeld University of Applied Sciences under the supervision of Anant Patel in cooperation with Andreas Tauch (Bielefeld University). Her research focuses on the development of encapsulation methods for cells (agricultural applications) and plant extracts (antimicrobial applications).

CALENDAR

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October	**Tablets by dry granulation** October 15, 2013 - Lyon, France
	Pan coating (workshop) October 15-17, 2013 - Binzen, Germany
	How to Make Microcapsules - Short Course October 23-25, 2013 - Henderson, NV, USA
	Microcapsule Tutorial and Update October 28-29, 2013 - Henderson, NV, USA
December	**28èmes Journées du GTRV s** December 2-4, 2013 - Orléans, France

PROGRAM 2014

February	**How To Make Nanocapsules** February 5-7, 2014 - Henderson, NV, USA
March	**6th Training School on Bioencapsulation** March 4-7, 2014 - Nha Trang, Vietnam
	Fluid bed processing March 11-13, 2014 - Binzen, Germany
	29th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology March 31 - April 3, 2014 - Lisbon, Portugal
May	**17th Industrial Microencapsulation Convention** May 2014 - Bruxelles, Belgium
July	**DynaCaps2014** July 15-18 , 2014 - Compiègne (France)
September	**22th International Conference on Bioencapsulation** September 2014 - Bratislava, Slovakia
November	**2nd South American Workshop on Microencapsulation** November 2014 - Joa Passao, Brazil

PROGRAM 2015

September	**20th Intenational Symposium on Microencapsulation** Septembre 2015 - Boston, USA

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JOB REQUESTS & POSITIONS

Postdoctoral Associate Position in Pharmaceutical Sciences

Midwestern University, College of Pharmacy

A two-year funded postdoctoral position is available immediately in the Department of Pharmaceutical Sciences. The successful candidate must have a Ph.D. in Pharmaceutical Sciences (or a closely related area) with strong emphasis on drug targeting and drug delivery. The candidate is also expected to possess excellent communication skills. Previous experience in grant writing is a plus. The main area of research will involve the preparation of lipid-based pharmaceutical nanocarriers like liposomes, micelles and nanoemulsions for the targeted delivery of biologically active molecules to mammalian mitochondria in order to develop novel cytoprotective and cytotoxic therapies. Of particular interest is the application of functionalized liposomes for mitochondrial antioxidant therapy and for the development of potential treatments of amyloid diseases.

Interested individuals should email a brief statement of past achievements, a C.V. and the names and contacts of three references to:

Volkmar Weissig, Sc.D., Ph.D.
Midwestern University, College of Pharmacy
Department of Pharmaceutical Sciences
19555 N. 59th Avenue, Glendale, AZ 85308, USA
vweiss@midwestern.edu

Vacancy: EngD student Measuring and Predicting the Burst Properties and Trigger Points of Microcapsules

Projetc description : Tailoring the burst properties of microcapsules containing active materials is of critical importance to ensure instantaneous release at the desired trigger point. In consumer product applications, a wide range of stress and strain versus time histories are observed, even for the same product use. To ensure optimal product design, it is proposed to measure the mechanical strength of microcapsules in relation to their chemistry, structure and environmental conditions, and to investigate fracture of microcapsules where two different substrates containing microcapsules relevant to industrial applications are rubbing together. The results obtained will be used to establish a model to predict the breakage of microcapsules under the varying conditions relevant to 'real world' consumer applications.

The work is very interdisciplinary in nature. The student should have very good knowledge of chemical engineering, mechanics, and material sciences, who will be trained in the skills of product formulation, microencapsulation, micromanipulation and tribology, which are available in the School of Chemical Engineering at Birmingham.

The student will be sponsored by School of Chemical Engineering at Birmingham and Procter & Gamble, UK.

Candidature

Zhibing Zhang, FIChemE
Professor of Chemical Engineering
University of Birmingham
Edgbaston, Birmingham B15 2TT, UK
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POST DOCTORAL POSITION Powder Technology, Gothenburg, Sweden

PowTech is an Initial Training Network (ITN) aiming to integrate interÐsectoral and multidisciplinary research in particle and powder technology into the training of 15 highly skilled young researchers, to strengthen the competitiveness of food and pharmaceutical industry and to strengthen the European Research Area. The people and the knowledge in powder technology to be developed in the PowTech network will contribute to the development of innovative products and effective powder processing in Europe by solving industrial problems and reducing knowledge barriers. The PowTech ITN network has 21 partners being of them 10 industrial companies (www.sik.se/powtech)

We are looking for 1 Experienced Researcher (ER) to be employed within PowTech ITN during a 1 year . The candidate will perform the research at SIK – The Swedish Institute for Food and Biotechnology located in Gothenburg Sweden, in collaboration with Wolfson Centre for Bulk Solids Handling, UK. The candidate is expected to have a doctoral degree (PhD) in Chemical Engineering, Food Engineering or a related subject, and preferentially experience in powder technology. According to Marie Curie rules, the ER will have an employment contract with SIK. The annual salary will follow the Marie Curie rules and is expected to be approximately 56 400 Ð/year plus travel and living allowance.

More information and candidature

Lilia.Ahrne@sik.se

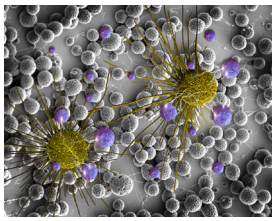
W3-Professorship of Biopharmacy and Pharmaceutical Technology, School of Pharmacy, University of Saarland (Campus Saarbruecken)

Saarland University School of Pharmacy is strengthened by the recently established Helmholtz-Institute for Pharmaceutical Research (www.helmholtz-hzi.de/hips). Candidate will be an international leading expert in biopharmacy and pharmaceutical technology, who will cooperate closely with the existing research groups and contribute her/his own cutting-edge research in this field. She/he is expected to contributeto the "NanoBioMed: Life and Matter" cluster. Strong participation in an ongoing master programme of pharmaceutical biotechnology («Wirkstoff-Biotechnologie») is expected. A close cooperation with partners from within the cross-border Saar-Lor-Lux Region and Wallonia as part of the "University of the Greater Region" (UGR) project is encouraged (www.uni-gr.eu).

Deadline for applications is 17.10.2013. Applicants should provide concise information and evidence regarding their individual scientific qualifications (including copies of degree certificates, a list of publications, reprints of their three most significant publications, evidence of external funding, summary of previous and planned research and teaching).

Complete applications should be addressed to

Prof. Dr. Volkhard Helms, Dekan der Naturwissenschaftlich- Technischen Fakultät III der Universität des Saarlandes, Campus Geb. C4.3, 66123 Saarbrücken, Germany, noting reference number W759.



Researchers at Penn State University, USA, target brain tumours

The production of monodispersed poly(lactic-co-glycolic) acid microspheres using an electrojetting technique for the targeted delivery of anti-cancer drug BCNU (carmustine or bis-chloroethylnitrosourea) has been developed by Mohammad Reza Abidian, assistant professor of bioengineering, chemical engineering and materials science and engineering at Penn State. The BCNU is unstable in vivo, has a half-life of only 15 minutes and has been successfully stabilized within microcapsules containing 4% w/w of the API. This early stage work is continuing and is applicable to a wide range of potential applications.

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Electro-spinning techniques applied to repair corneas

The development of a Poly d,l-lactide-co-glycolide (PLGA) membrane from electrospun fibres was described in the paper "Simplifying corneal surface regeneration using a biodegradable synthetic membrane and limbal tissue explants". Project participants included the University of Sheffield, UK, L V Prasad Eye Institute, India and The Electrospinning Company Ltd, UK:
http://www.electrospinning.co.uk/
Success would lead to less reliance on human tissue banks and specialist culture laboratory supply making treatment more accessible.

The paper can be found at:
http://bit.ly/1ezH2ci
and a recent review of electrospinning techniques has been published by Suwan N. Jayasinghe of University College London:
http://rsc.li/16z8i2J

creathes

Creathes launch CREASPHER

Following 18 months in development Creathes of Belfort, France
http://www.creathes.com/
have launched their new microencapsulation platform technology Creaspher aimed at cosmetics, agrochemical and food industry applications. Via Formule Verte

More information
http://bit.ly/16zc1gt

FormuleVerte.com

PEPSICO

Releasably encapsulated aroma

As widely reported, PepsiCo has applied for a patent on providing encapsulated flavours for packaging, where flavour is released at point of use, presenting a preferred flavour impact to enhance the consumer experience. The recently published application (WO 2013032631 A1) can be found at the WIPO website
http://bit.ly/16qA7ZO

Biomedal

Cellena® Microencapsulator is based on the immobilization of cells within a semipermeable membrane that protects them from mechanical stress and immunological responses, allowing the bidirectional diffusion of nutrients, oxygen and secretory products including waste. Cellena® is an user-friendly equipment for biotechnological research based on the Flow Focusing® technology developed by Ingeniatrics S.L. Cellena® allows homogeneous encapsulation of cells/organisms under sterile conditions and selecting the particle size as required by each specific application.

More information
http://bit.ly/18VPUku

ENCAPSYS®
MICROENCAPSULATION

$1.2 million to improve nanomanufacturing

Engineers at Ohio State University, USA have been awarded a four-year $1,174,000 grant from the National Science Foundation (NSF) to establish new methods and equipment for the continuous manufacturing of nanoparticles using electrospray technology. (via Nanowerk website)

Read more at:
http://bit.ly/GAnqGR

GTR

GTR changes name in Société Française de Nanomédecine (SFNano)

You may propose ideas for new logo on
http://bit.ly/GACzY3

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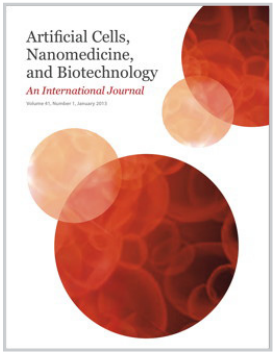

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INTRODUCTION

The 2013 General assembly held at the Annual Conference in Berlin, Germany on August 29, 2013. All participants at the conference were invited to attend the General Assembly.

2012 ACTIVITY REPORT

Five issues of the BRG newsletter were published in 2012 and sent by email to 7000 persons.

- One short issue in January
- **Encapsulation in Cosmetics,** special issue, in March, with the support of Dr Eric Perrier from LVMH.
- **Polyelectrolytes for Encapsulation** in June, with the help of Prof. Christine Wandrey from EPFL.
- **Tissue engineering & Encapsulation** in October with the help of Prof Paul de Vos from UMCG.
- **Best student contributions** in December, prices awarded at the XXth ICB, Orillia.

Three events were organized in 2012:

- 15th Industrial Symposium and 6th Trade Fair on Microencapsulation, held in Archamps, France, March 20-22
- First South America Workshop on Microencapsulation, held in Limeira, Brasil, April 30 - May 2, mainly organized by Prof. Ana Prata Soares
- And XXth International Conference on Bioencapsulation, held in Orillia, Canada, septembre 21-24, organized by Prof. Ronald Neufeld.

The 3 events were very successful both in term of attendance and quality of the contributions (See table 1)

FINANCIAL REPORT

The accounting of Limeira meeting was handled directly by the UNICAMP (except for a few registrations and expenses). Table 2 and 3 provide a summary of the incomes and expenses for the events held in Archamps and Orillia. The following general observations may be done:

Table 1	Participants					Contributions		
	Industrials	Researchers	Students	Exhibitors	Total	Orals	Posters	Grants
Archamps	77	13		16	106	12		12
Limeira	15	70	50	5	140	26	50	50
Orillia	22	55	45	3	125	40	40	65

- For Limeira, one has to point out a very large Brasil public support of 27 000 €
- Archamps Symposium was profit-making: 49 571 €.
- Orillia conference budget was negative (14 470 €) but mainly due to the grants (17 344 €).

Table 4 provides the accounting for the association restricted to operations carried out in 2012. It results in a positive balance for 2012 of 32660 €, leading to an increase of the BRG financial reserve to 76 214 €.

The 2012 accounting was externally audited and the General Assembly approved unanimously the financial report.

STEERING COMMITTEE

The General Assembly has unanimously elected until the next General Assembly to be held in September 2014:

- Denis Poncelet as President
- Thierry Vandamme as Secretary
- Ronald Neufeld as Treasurer

The Steering Committee will be completed by a group of people taking responsibility for one of the BRG events or newsletter in 2013 and 2014. Especially, Paul de Vos and Michael Whelehan as Chief Editors for the newsletter.

2013-2014 ACTIVITIES

Three events have been organized in 2013:

- 16th Industrial Symposium and 7th Trade Fair on Microencapsulation, held in Madison, WI, USA, June 25-27, co-organized by Dr Don Josefchuck
- 5th Training School on Bioencapsulation, held in Nantes, France, April 9-12
- And XXI International Conference on Bioencapsulation, held in Berlin, Germany, August 28-30, organized by Prof. Stephan Drusch and Dr Thorsten Brandau.

Four events are in preparation for 2014:

- 6th Training School on Bioencapsulation, held in Nha Trang, Vietnam, March 4-8
- 17th Industrial Microencapsulation Convention (formally Industrial Symposium and 7th Trade Fair) to be held in Brussels, Belgium, in May, organized by Dr Jean Paul Simon
- 22th International Conference on Bioencapsulation, held in Bratislava, Slovakia, in September, organized by Prof. Igor Lacik.
- 2nd South America Workshop on Microencapsulation, held in Joa Passao, Brasil, in November, mainly organized by Prof. Ana Luisa Braga

Four issues of the BRG newsletter are scheduled in 2013

- **Microencapsulation by chemical methods** in March, co-edited by Prof. Bojana Boh and Dr. Yves Frère
- **Microfluidcis and microencapsulation** in June, on co-edited by Dr Raul Rodrigo Gomez and Johan Smet from P&G
- **Best student contributions** in october, prices awarded at the XXIth ICB, Berlin.
- **Enzyme Microencapsulatio** in December, co-edited by Prof Luis Fonseca and Dr Gabrie Meesters

CLOSING

As no question were raised by the participants, the General Assembly was closed by the President.

Table 2 : 2012 Archamps symposium	
Registration	85 003 €
Exhibitors	18 800 €
Reception	-23 587 €
Management	-13 515 €
Printing	-10 631 €
Divers	-6 499 €
Balance	49 571 €

Table 3 : 2012 Orillia conference	
Registration	64 737 €
Sponsoring	5 700 €
Reception	-31 922 €
Grants	-17 344 €
Management	-17 048 €
Divers	-12 348 €
Balance	-8 225 €

Table 4 : 2012 Orillia conference	
2012_Archamps	49572 €
2011 Amboise	1632 €
2012_Limeira	-1257 €
2012_Orillia	-14471 €
2012_BRG	-2816 €
Balance	32660 €

Bioencapsulation Research Group

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Bioencapsulation Research Group is a non-profit association promoting networking and research in the encapsulation technology of bioactives. It organises academic conferences and industrial symposiums, publishes newsletters and manages a website.

More information : http://bioencapsulation.net

KEEP CONTACT BY REGISTERING ...

Registration is based on a voluntary annual fee. If you wish to simply receive the newsletter and be advised about future events, register online at: http://bioencapsulation.net

Be an active member pay the registration fee and get more services

- Reduced registration fees to BRG events
- Full access to conference proceedings (› 1700)
- Access to the forum and internal mailling
- Possibility to contribute to the newsletter
- Reduction for the conference registration
- Priority for awarding of conference grants

Class	Annual fees
Industry members	100 €
Researchers [1]	60 €
Students [2]	30 €
Honorary member and corporate registration [3]	1000 €

[1] public and non-profit organizations, contact us for group registration
[2] registered for a master or PhD program, less than 30 years old.
[3] Open access to 1 full page in 1 issues (1/2 page in 2 issues ...) in the newsletter
Registration fees may be paid by credit card (preferably), bank transfer or cheque.

For more information or an invoice, see the registration page on http://bioencapsulation.net

Thanks to **Agence I** (http://www.agence-i.eu/) for designing the newletter. **Geraldine Brodkorb** (gbrodkorb@eircom.net) for English corrections,Brigitte Poncelet (http://impascience.eu) editing corrections and the editorial board for their help.

REGISTRATION DATA

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- First name: ..... Last name: .....
- Affiliation: ..... Department: .....
- Address: ..... Address (cont.): .....
- Zipcode: ..... City: .....
- State: ..... Country: .....
- Phone: ..... Fax: .....
- Email: ..... Website: .....
- Password: ..... Repeat password: .....
- Registration class: ..... Registration fees: ..... €

Send your registration to : Bioencapsulation Research Group 5 rue de la maison blanche44240 Sucé sur Erdre France