AQUEOUS TWO-PHASE SYSTEMS FOR THE PRODUCTION OF A NOVEL TYPE OF MICROCAPSULES

V.Breguet[1] , V.Vojinovic, U.von Stockar, I.W. Marison[2] *
[1] LGCB, Ecole Polytechnique Fédérale - Lausanne, Switzerland
[2] Glasnevin 9, Dublin City University – Dublin 9, Ireland (ian.marison@dcu.ie)

Introduction

Due to the limitations of alginate-PLL capsules for mammalian cell immobilisation (weakness of the membrane, presence of intracapsular alginate), a novel type of aqueous-core microcapsules was developed, based on aqueous two-phase systems (ATPS). The fact that two or more phases form in an aqueous medium through the addition of incompatible polymers, or polymer and salts, is known for over one hundred years. Since alginate is already widely used for many microencapsulation applications (Thu 1995) , and forms hydrogels suitable for constituting the membrane, the challenge consists in finding another salt or polymer forming an ATPS with this polysaccharide (Antonov 1980). The thermodynamic incompatibility of proteins and polysaccharides has been known for a long time (Simeone 2004), and the most studied ATPS involving alginate contains casein as a protein.

The principle of ATPS may be extended in order to produce aqueous-core microcapsules, using the co-extrusion jet break-up technique, as the encapsulation device can be fitted with a concentric nozzle. Using two non-miscible aqueous solutions, the first one can be extruded through the inner nozzle, and the other solution, constituting the membrane, through the external nozzle. Applying a frequency on the nozzle would then allow breaking the jet, leading to the formation of aqueous-core microcapsules in a single step procedure.

Material and Methods

Phase Diagram: Aqueous solutions of Na-Alginate (Inotech, Basel, Switzerland) and Na-caseinate (Acros, Geel, Belgium) were prepared (pH 7), concentration range was 1.5-7% (w/v) for alginate and 2.5-10% (w/v) for casein. Five mL of pure component solutions were blended together, and allowed to equilibrate for 48 hours at room temperature. The volume of each phase was measured, and the concentration of both alginate and casein were assessed according to the methods described below. For alginate quantification, 16 mg of DMMB (Sigma-Aldrich Chemie, Steinheim, Germany) were dissolved in 5 mL of 100% ethanol. In 900 mL deionized distilled water, 2.37g of NaCl and 3.04g of glycine were dissolved and mixed with DMMB solution, pH was adjusted to 3 and volume completed to 1 liter with water. The DMMB assay consists in mixing 10 µL of sample with 1 mL of reagent solution and to measure the absorbance with a UV-VIS spectrophotometer (Uvikon 810, Tegimenta AG, Rotkreuz, Switzerland) at 658 nm, with water as reference. Casein content was determined using the Bradford Assay (Bio-Rad, München, Germany).

ATPS capsules: The encapsulation device (Inotech, Basel, Switzerland) was fitted with a concentric nozzle (300/200µm outer / inner diameter). Sterile casein 7.5% w/v (dissolved in 10 mM MOPS, pH 7, 0.85% NaCl) solution was extruded (4.5 $mL \cdot min^{-1}$) through the central nozzle and sterile alginate 3%w/v solution (dissolved in 10 mM MOPS, pH 7, 0.85% NaCl) through the external one (9.5 $mL \cdot min^{-1}$), applying a frequency of 502 Hz. The capsules were then collected in a gelling bath containing 10mM MOPS pH 7, 200mM $CaCl_2$, and were incubated for one month in an isotonic buffer (10 mM MOPS, pH7, 0.85% NaCl, 0.1% NaN_3). Capsule size determination was carried out using a microscope (Axiolab, Carl Zeiss Jena GmbH, Jena, Germany) connected to a digital video camera (Sony CCD Iris Camera, Sony Corporation, Tokyo, Japan) and software for image analysis (Cyberview Cervus International, Courtaboeuf, France). The mechanical resistance of capsules was determined using a Texture Analyzer (TA- 2xi, Stable Micro Systems, Goldaming, UK). Measurements were carried out on a monolayer of capsules spread on a glass microscope slide.

Results and Discussion

Mathematical modeling

The model presented is based on the virial expansion theory, and applied to predict the alginate-casein phase diagram, with pure water as solvent (no salt added). Let us define a ternary system, composed of two solutes and a solvent. In this particular case, solutes are alginate (ag) casein (cas), and the solvent is water (w). The condition that defines the spinodal curve (i.e. miscibility gap), at constant temperature and pressure, separating regions of absolute instability is (Edmond, 1968):

$$\frac{\partial^2 \Delta G_{mix}}{\partial m_i^2} \leq 0$$

According to the expressions defining the chemical potential for alginate and casein, and water with the virial expansion theory, the spinodal curve is then given by the following expression:

$$R^2 \cdot T^2 \left(\frac{1}{m_{ag} m_{cas}} + \frac{a_{cas/cas}}{m_{ag}} + \frac{a_{ag/ag}}{m_{cas}} + a_{cas/cas} a_{ag/ag} - a_{ag/cas}^2 \right) = 0$$

where

$$a_{ag/ag} = \frac{2M_{ag}^2 \tilde{B}_{ag/ag}}{\rho \cdot 1000^2}; a_{cas/cas} = \frac{2M_{cas}^2 \tilde{B}_{cas/cas}}{\rho \cdot 1000^2}; a_{ag/cas} = \frac{2M_{ag} M_{cas} \tilde{B}_{ag/cas}}{\rho \cdot 1000^2}$$

B values for alginate and casein were found in the literature (Semenova 2007, Zhang 1997), and put in the previous model to get the spinodal curve ($\widetilde{B}_{ag/ag} = 1.0 \cdot 10^{-3}$ mol·cm^3·g^{-2}, $\widetilde{B}_{cas/cas} = 2.7 \cdot 10^{-4}$ mol·cm^3·g^{-2}). Casein and alginate molecules were approximated as two rigid spheres, and then used to estimate $\widetilde{B}_{ag/cas} = 1.1 \cdot 10^{-3}$mol· cm^3·g^{-2}.

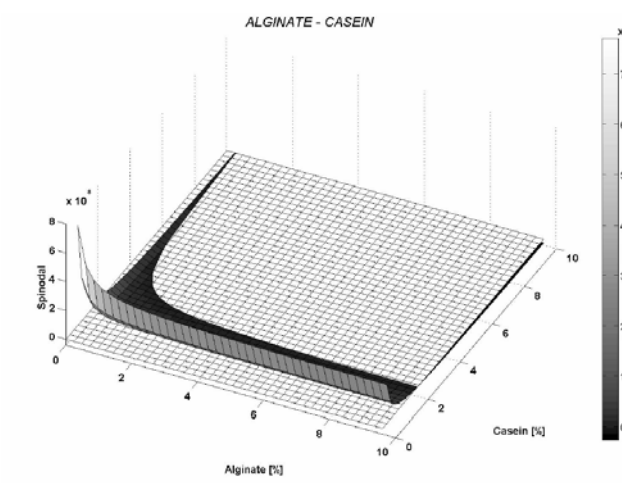

Figure 1: Determination of the spinodal curve for alginate-casein solution as a function of polymers concentrations

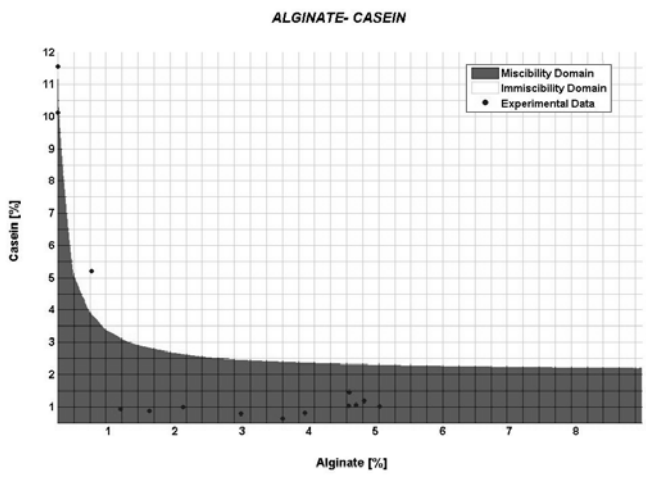

Figure 2: Comparison of the spinodal curve for alginate-casein solution an experimental data as a function of alginate and casein concentrations

Since second virial coefficients found in the literature were defined for alginate and casein from other suppliers (i.e slightly different molecular weights, other M:G composition for alginate), this may explain the slight difference between experimental and theoretical results. The limit of miscibility calculated with the simulation was then compared with data obtained experimentally (Figure 2). Practical data and the theoretical approach are in good agreement, despite the assumptions (electrostatical interactions neglected, polymer molecules considered as spheres).

ATPS Capsules

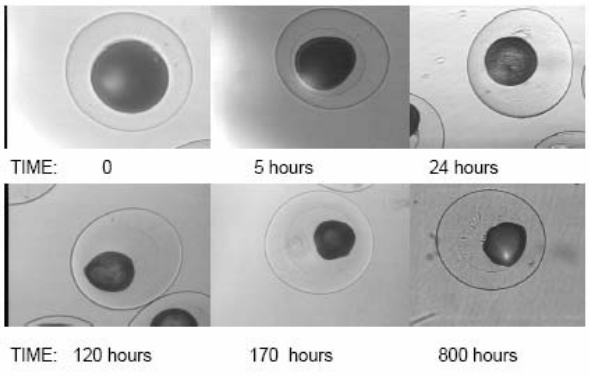

Figure 3: Alginate-Casein microcapsules morphology as a function of time. External capsule radius =340 µm

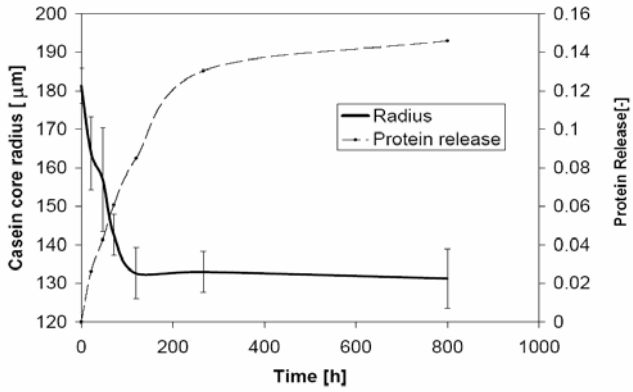

Figure 4 : Casein core radius (left axis) and casein release as a function of time (right axis, absorbance unit)

The resulting alginate-casein capsules had an external radius of 340 µm, and a membrane thickness of 100 µm. Size distribution was very narrow, with less than 5% standard deviation. The radius and membrane thickness remained constant during the entire month of incubation in isotonic buffer, proving the good integrity and robustness of these capsules. However, Ca^{2+} is known to induce caseinate precipitation, due to binding of the cation to the protein (Alvarez 2007). The concentration of calcium in the gelling bath being 200mM and casein concentration being 75 $g \cdot L^{-1}$, the formation of aggregates of casein in the core of the capsule which occurred during the capsules formation might thus be explained. The pictures (Figure 3) show that the casein in the core diffuses as a function of time, since the black surface in the core decreases. This diffusion was confirmed by casein release to the isotonic surrounding medium (Figure 4). After one month of incubation, the alginate membrane was dissolved (with citrate buffer), to analyze the casein core remaining in the core of the capsule. The residual casein was found to be a solid (gel-like) state.

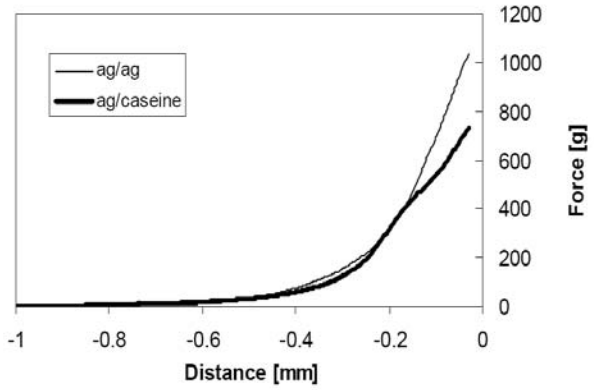

Figure 5: mechanical resistance as a function of the compression distance for alginate beads (ag/ag) and ag-casein capsules (radius=340µm)

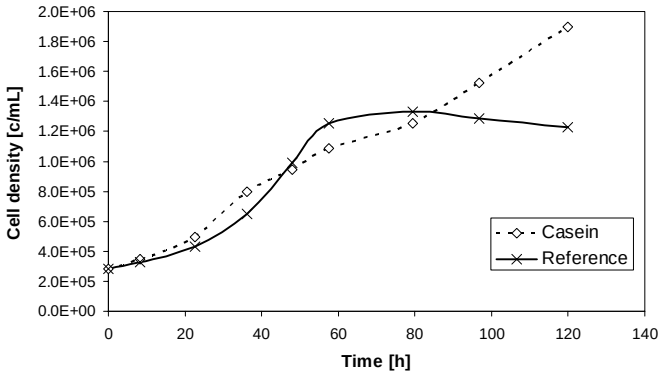

Figure 6: Batch culture of CHO cells in suspension in CHOmaster HP-1 medium; reference and with 3.5% casein

Mechanical resistance was also measured throughout the incubation experiment, and also remained constant over time. Under compression, aqueous-core capsules are crushed but do not actually burst (Figure 5).

Batch cultures of CHO cells in the presence of casein indicated that the protein does not affect cell growth (Figures 6). The growth curve in the presence of casein presented a first exponential growth phase (0-50 hours) and then a pseudo-linearly growth phase (50-120 hours), while the reference culture followed a first exponential growth phase and then a plateau. Final cell concentration was 40% higher in the culture with casein.

Conclusions

The results showed the good agreement between experimental data and the theoretical prediction of aqueous two-phase systems using the osmotic virial expansion theory, despite the numerous assumptions (electrostatical interactions neglected, polymer molecules considered as spheres). This suggests that predictions of phase diagrams with other polymers is feasible, providing that the second virial coeffcient of pure polymer solution in water is known. The miscibility and imiscibility domain of alginate-casein mixtures as a function of their concentration in water could thus be established. The aqueous two-phase alginate-casein system led to the successful production aqueous-core microcapsules, using the jet break-up technique with a concentric nozzle. The radius was 340 µm, and alginate membrane thickness was 100µm. The resulting capsules were mechanically stable over one month of incubation in isotonic buffer, did not burst under compression and thus resembled to alginate beads. Batch cultures of CHO cells in the presence of casein showed excellent biocompatibility. ATPS-microcapsule is thus a promising technique for mammalian cell encapsulation, overcoming a number of limitations associated with alginate-PLL capsules.

List of Abbreviations

$\widetilde{B}$	Second virial coeffcient ($mol \cdot m^3 kg^{-2}$)
G	Molar Gibbs free energy ($J \cdot mol^{-1}$)
M	Molar mass ($g \cdot mol^{-1}$)
T	Temperature (K)
R	Universal gas constant ($J \cdot mol^{-1} \cdot K^{-1}$)
m	Molality ($mol \cdot kg^{-1}$)
ρ	Density ($kg \cdot m^3$)

References

1. E.M. Alvarez, P.H. Risso, Gatti C.A., M. Burgos, and V. Suarez Sala (2007). *Calcium induced aggregation of bovine caseins: effect of phosphate and citrate*. Colloid & Polymer science, 285:507-514.
2. Y.A. Antonov, V. Grinberg, N.A. Zhuravskaya, and VB. Tolstoguzov (1980). *Liquid two phase water-protein-polysaccharide systems and their processing into textured protein product*s. Journal of Texture Studies, 11:199-215.
3. E. Edmond and A.G. Ogston (1968). *An approach to the study of phase separation in ternary aqueous systems*. The Biochemical Journal, 109:569-576.
4. M.G. Semenova (2007). *Thermodynamic analysis of the impact of molecular interactions on the functionality of food biopolymers in solution and in colloidal systems.* Food Hydrocolloids, 21:23-45.
5. M. Simeone, A. Alfani, and S. Guido (2004). *Phase diagram, rheology and interfacial tension of aqueous mixtures of Na-caseinate and Na-alginate*. Food Hydrocolloids, 18(3):463- 470.
6. B. Thu, P. Bruheim, T. Espevik, O. Smidsrød, P. Soon-Shiong, and G. Skjåk-Bræk (1995). *Alginate polycation microcapsules : I. interaction between alginate and polycation*. Biomaterials, 17:1031-1040.
7. H.C. Zhang, H.H. Zheng, Q. Zhang, S. Gao, and J. Wang (1997). *Studies on the second virial coeffcient of sodium alginate solution.* Polymers for Advanced Technologies, 8:722-726.