1- vs 2-step protocols result in different properties of SA−CS/PMCG microcapsule

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Introduction

Polymeric capsules represent a sophisticated material finding various application and many diverse areas. Among all the materials, the most frequently used principle for microcapsule formation is that based on the polyelectrolyte complexation. This is true especially for the applications in biomedicine and biotechnology due to fast microcapsule formation process under mild and physiological conditions (Lacík 2005). Among a number of microencapsulation systems, the capsule based on combination of polyanions, sodium alginate (SA) and sodium cellulose sulfate (CS), interacting with polycation poly(methylene-co-guanidine) (PMCG) developed for encapsulation of pancreatic islets (Lacik 1998) has found its important place.

Throughout the years, two encapsulation protocols for encapsulation of biological material in the SA−CS/PMCG capsules have been employed. The originally developed SA−CS/PMCG capsule was made by a 1-step protocol (Lacik 1998) using the combination of polyelectrolyte complexation and ionotropic gelation in one step. The specificity of this capsule formation is fast reaction ranging a few tens of seconds, which requires a precise control of reaction time achieved by employing the multiloop reactor (Anilkumar 2001). The second protocol is the 2-step process (Renken 2007). In the first step the SA−CS droplets are ionically crosslinking in the receiving bath containing a multivalent cation (e.g. Ca^{2+}, Ba^{2+}) to create the SA−CS beads. In the second step, the beads are cured in the solution of PMCG forming the SA-CS/PMCG capsule.

The chemistry of 1- and 2-step processes is at glance identical, however, a difference between the properties and stability of these microcapsule types can be expected due to different gelling sequences (Lacík 2006). CS chains do not interact with Ca^{2+} ions. Therefore, they may leach out the SA−CS bead during the bead formation, storage and washing steps before their stabilization by PMCG in the 2nd step. CS is immediately stabilized by PMCG in the 1-step process.

The aim of this contribution is to discriminate between the SA-CS/PMCG microcapsules prepared by either 1- or 2-step protocols. The techniques used in this study were optical microscopy, compression test, inverse size exclusion chromatography and confocal laser scanning microscopy.

Materials and methods

Chemicals. High viscosity sodium alginate with 60 % of mannuronic acid units from ISP Alginates and sodium cellulose sulfate from Acros Organics were used as polyanions. Poly(methylene-*co*-guanidine) hydrochloride (PMCG) was purchased from Scientific Polymer Products Inc. (USA). Rhodamine 123 from Molecular Probes-Invitrogen was used as the fluorescent label.

Capsule formation. The concentrations of components are contained in Table 1. The flow rate of SA-CS solution was about 0.6 ml/min. In the 1-step process, the polyanion droplets were collected for 5 s in 20 ml of cationic solution containing 1.2 wt.% PMCG, 2.0 wt.% $CaCl_2$ and 0.9 wt.% NaCl at p*H* 7.5 and microcapsules were let to form for 40 s. The reaction was stopped by washing the microcapsules with saline solution. In the 2-step process, the polyanion droplets were collected for 5 s in 20 ml of 2.0 wt.% $CaCl_2$ and 0.9 wt.% NaCl at p*H* 7.5 with gelling time of 40 s. The reaction

was stopped by immediate 3 washing steps of (SA-CS)/Ca^{2+} beads in saline solution. Beads were placed to 20 ml of solution containing 1.2 wt.% PMCG and 0.9 wt.% NaCl at p*H* 7.5 for 40 s followed by their wash in saline solution. This protocol enabled to prepare small batches of capsules of the same size of initial SA−CS droplets for both 1- and 2-step protocols. For determination of molecular weight cut-off by the inverse size exclusion chromatography, 10 ml of each type of microcapsules were prepared using the multiloop reactor (Anilkumar 2001) filled with polycation solution in the 1-step process and $CaCl_2$ saline solution in the 2-step process, respectively. The number of loops and flow rate of PMCG solution were selected to provide the reaction time of 40 s. Reaction was stopped by collecting microcapsules at the exit of the reactor in an excess of saline solution in the 1-step and water in the 2-step process. Using saline solution in the latter case gave such a critical stickiness of microcapsules that they could not be further processed. Also the stickiness was the reason that 2 wt. % $CaCl_2$ concentration had to be used instead of typically used 1 wt. % $CaCl_2$. In the 2-step protocol, microcapsules were immediately washed in distilled water followed by formation of the membrane in PMCG solution for another 40 s. In both protocols, 50 mM sodium citrate in saline solution was applied for 10 min to equilibrate the membrane composition. A stainless steel sieve was used to remove and wash the microcapsules. Microcapsules were stored in a refrigerator in saline solution containing 200 ppm of NaN_3.

		Polyanion solution in 0.9 wt.% NaCl		Polycation solution in 0.9 wt.% NaCl	
		SA(wt.%)	PMCG wt.%)	PMCG (wt.%)	$CaCl_2$ (wt.%)
1-step protocol		0.9	0.9	1.2	2.0
2-step protocol	1st step	0.9	0.9	−	2.0
	2nd step	−	−	1.2	−

Table 1. Composition of solutions used for* microcapsule *preparation by 1- and 2-step protocols.

Inverse size exclusion chromatography (ISEC). ISEC was used for determination of the molecular weight cut-off (MWCO) of microcapsules (Briššová 1996). Narrow distributed pullulan standards (Polymer Laboratories) of concentration 3 mg/ml were injected onto a glass column 10×250 mm (Omnifit) fitted with an adjustable plungers on each side and filled with 10 ml of microcapsules for these experiments. The column was attached to the SEC set-up. A 0.9 wt. % NaCl solution containing 0.02 % NaN_3 was used as an eluent at a flow rate of 0.2 ml/min.

Optical microscopy. An optical microscope (Kvant) equipped with a color CCD camera (Mintron) and a software Prover Image Forge v1.1 (Prover s.r.o.) were used for taking the microcapsule images and their evaluation. About twenty-five microcapsules per batch were randomly selected to determine the microcapsule average size and membrane size.

*Mechanical properties. C*apsules were tested in the compression resistance test using a Texture Analyzer TA-2Xi (UK) by compressing individual microcapsules. The force exerted by the probe on the capsule was recorded as a function of compression distance at the deformation speed of 0.5 mm/s. Twenty five capsules per batch were analyzed in order to obtain statistically relevant data.

CLSM. Microcapsules were labeled with various fluorescence labels at the concentration of ~10^{-7} mol.L^{-1} in saline solution and were left to equilibrate for 60 min. For this presentation, data with Rhodamine 123 of were selected. The laser scanning confocal microscope LSM510 META on Axiovert 200 (Zeiss) using 40x/1.2W C-Apochromat objective was used.

Results and discussion

The optical images of SA-CS/PMCG capsules prepared by on 1- and 2-step processes are shown in Figure 1. The capsules prepared by 1-step process are optically transparent whereas the 2-step microcapsules are darker with a slightly precipitate-like core.

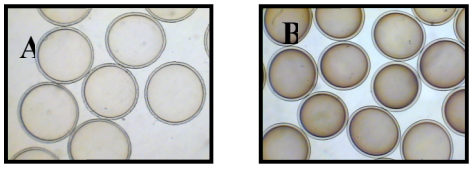

Figure 1. Optical microscopy images of SA-CS/PMCG capsules prepared by 1-step (A) and 2-step (B) processes.

Protocol	Size (μm)	Membrane thickness (μm)	Compression resistance (g/microcapsule)	MWCO to pullulans (kDa)
1-step	970 ± 30	37 ± 3	5.9 ± 2.6	10
2-step	870 ± 30	36 ± 4	12.2 ± 4.7	60

Table 2: Characteristics of SA−CS/PMCG capsules prepared by 1-step and 2-step processes

Table 2 summarizes the quantities obtained for both SA−CS/PMCG preparation protocols. Apart from the clear differences seen immediately in Figure 1, these two capsules are rather different also with respect to other characteristics. The size of 2-step capsules is smaller than of 1-step ones. Since the size of SA−CS droplets was in both cases the same, a difference in size of capsules results most likely from the extent of gelling. As the membrane thickness is the same for both capsules, the volume of membrane in case of 2-step capsule is lower than that of the 1-step capsule. What can be the reason for a lower complex volume, decreased capsule swelling and higher extent of CS−PMCG interactions resulting in precipitate-like complex formation for the 2-step process capsule ?

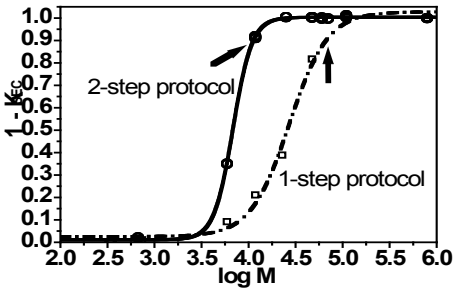

Figure 2. Dependence of partition coefficient on molecular weight for pullulan standards in estimating the calibration curve for the column filled with capsules. Arrows point at the MWCO values for respective capsules.

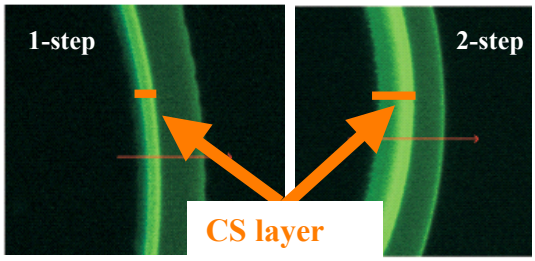

Figure 3. CLSM visualization of capsule membrane formed by 1- and 2- step protocols by Rhodamine 123. The thickness of the CS layer in each capsule type.

These observations indicate that the density of membrane prepared by the 2-step process is higher than that for the 1-step process, which is supported by values of compression resistance, being twice higher for the 2-step capsule, and a large difference between the MWCO values. The 2-step capsule is having substantially decreased MWCO value of 10 kDa compared to 60 kDa determined for the 1-step type of SA−CS/PMCG capsule. This is demonstrated in Figure 2 showing the results from the inverse size exclusion chromatography for both capsules indicating the MWCO values. In order to understand the reason behind this behavior, we applied confocal laser scanning microscopy to visualize the distribution of polymers in the membrane and capsule. Capsules were labeled by

diffusion of the charged fluorescent labels to the capsule interior. The labels interact by the electrostatic interactions with the residual opposite charges of the polyelectrolytes used for the formation of capsules. Figure 3 shows one of the results obtained with Rhodamine 123, which was found to predominantly bind to CS. While for the 1-step process CS layer forms a thin rim located at the interface between the core and the membrane (J. Podskočová et al. (2005), in the 2-step process this rim is much thicker and penetrates significantly into the capsule membrane. This visualizes the CS molecules which have penetrated in the outward direction during the Ca^{2+} gelling step and washing steps before being stabilized by PMCG diffusing the bead interior during the 2nd step. This is most likely responsible for a higher density of CS chains in the membrane volume creating the conditions for seeing (i) the precipitate-containing capsules, (ii) smaller microcapsule size, (iii) lower membrane volume, (iv) higher mechanical strength, and lower permeability in case of 2-step capsules compared to the 1-step one.

Conclusions

Although the chemistry between 1- and 2-step processes is identical, data presented in this contribution confirmed that there is a considerable difference between the properties of the SA-CS/PMCG capsules prepared by these different protocols. The first point to stress is that we have also been able to prepare the SA-CS/PMCG capsules by the 2-step process, which do not exhibit precipitation as reported in e.g. ref.6. As the second point we would like to emphasize that although at glance the 2- step protocol may appear as a suitable way to increase mechanical resistance and decrease MWCO, following this direction in capsule optimization is not straightforward and will likely result in irreproducible data because of high sensitivity to preparation conditions. During gelling and washing phase, this capsule may be seen as "alive" in terms of the CS distribution, which position is fixed only after exposure to PMCG. Therefore groups preferring the 2-step protocol should keep this information in mind in order to ensure the reproducibility especially when preparing large batches of capsules with long times before exposing the capsules to PMCG solution.

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