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RESEARCH PAPER

Lyophilization of Protein-Loaded Polyelectrolyte Microcapsules

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ABSTRACT

Purpose To evaluate if lyophilization can be used to obtain a dry formulation of polyelectrolyte microcapsules, which have emerged as a new class of microparticles for the encapsulation and delivery of biomacromolecules.

Methods Microcapsules composed of dextran sulfate and poly-L-arginine were obtained by coating $CaCO_3$ microparticles by means of the layer-by-layer technique. Microcapsules were lyophilized using different stabilizers; intactness was checked by CLSM and SEM. Horseradish peroxidase was encapsulated as model enzyme and retained activity after freeze-drying was determined using a fluorescence assay. Ovalbumin was encapsulated as model antigen; immunogenicity after lyophilization was evaluated *in vitro* by a T-cell proliferation assay and *in vivo* by measuring the antibody titer in mice.

Results The results clearly demonstrate the necessity of using polyols in the formulation to prevent rupture of the microcapsules and to preserve the activity of encapsulated enzymes. Lyophilized microcapsules appeared as a promising adjuvant for antigen delivery, as both *in vitro* as *in vivo* assays showed higher immune activation compared to free antigen.

Conclusions Lyophilization is a promising strategy towards improved stability of protein-loaded microcapsules.

KEY WORDS drug delivery · encapsulation · layer-by-layer · lyophilization · microcapsules

ABBREVIATIONS

DEXS	dextran sulfate
HRP	horseradish peroxidase
LbL	layer-by-layer
OVA	ovalbumin
pARG	poly-L-arginine

INTRODUCTION

Over the last decade there has been a growing interest in microparticles made by the so-named layer-by-layer (LbL) technique (1). This technique is based on the sequential adsorption of oppositely charged polyelectrolytes onto the surface of charged colloidal particles which are used as templates (2,3). Not only have synthetic polymers (2,4,5) and biopolymers (6–8) served as layer constituents, but enzymes (9), nucleic acids (6,10), lipids (11) and nanoparticles (12,13) have also been incorporated in LbL shells (14). Furthermore, different types of templates have been

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considered, including biological cells (15), drug crystals (16,17), organic (18–20) and inorganic (21,22) colloidal particles. An interesting feature offered by some of these templates is the possibility to obtain so-named 'hollow LbL capsules' by dissolving the template after LbL assembly, as illustrated in Fig. 1. The LbL capsules thus obtained may hold promise for a variety of applications, such as micro-reactors (23) or drug delivery systems (24–26).

$CaCO_3$ microparticles are well investigated as templates for the preparation of hollow LbL capsules (7,8,27). As shown in Fig. 1, $CaCO_3$ microparticles can be loaded with, e.g., therapeutic proteins by co-precipitation which involves capturing of the proteins in the $CaCO_3$ microparticles during the formation of the latter (28). Subsequently, an LbL shell is deposited on the $CaCO_3$ cores, which are, in a next step, dissolved by adding EDTA. Besides their high loading capacity for proteins (paper in press), $CaCO_3$ templates offer the additional advantage that both the preparation and dissolution of the $CaCO_3$ particles occur in aqueous conditions that barely affect the biological function of the encapsulated proteins. A variety of enzymes has been encapsulated in LbL capsules, e.g., urease (29), α-chymotrypsin (28,30), catalase (31), glucose oxidase (32,33) and peroxidase (32,33).

LbL capsules may hold potential as drug delivery vehicles, especially as carriers for antigens for the purpose of vaccination. Our group has studied the cellular uptake of LbL capsules, their enzymatic degradation by proteases and biocompatibility both *in vitro* (7) and *in vivo* (34). We also showed the *in vivo* potential of polyelectrolyte microcapsules as antigen carriers for vaccination via different routes (35).

Polyelectrolyte microcapsules dispersed in an aqueous medium are highly stable, due to the many electrostatic interactions between the oppositely charged polyelectrolytes which keep LbL shells intact. As a consequence, dispersions of polyelectrolyte microcapsules can be easily stored for long periods of time. However, proteins encapsulated in the aqueous lumen of the LbL microcapsules are physically and/or chemically unstable, which presents a serious challenge for the use of LbL capsules as protein delivery vehicles. In the pharmaceutical field, lyophilizing protein solutions is a common procedure to improve the stability and thus the shelf-life of therapeutic proteins (36). To our knowledge, lyophilization of LbL microcapsules has never been studied before, although it represents a crucial step towards their practical applicability as drug delivery vehicles. With the aim to develop a dry formulation of LbL capsules, the major objective of the present work is to answer the question whether (protein-loaded) polyelectrolyte microcapsules survive the freezing stress during lyophilization. The structural features of the microcapsules were studied with the aid of confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). We also investigated whether horseradish peroxidase, encapsulated as a model protein in the polyelectrolyte microcapsules, retains its enzymatic activity after lyophilization of the microcapsules. Furthermore, we encapsulated ovalbumin (OVA) as model antigen in polyelectrolyte microcapsules and assessed the immunogenicity of OVA-loaded microcapsules, before and after lyophilization, both *in vitro* by a T-cell proliferation assay and *in vivo* by measuring the antibody concentration in sera of immunized mice.

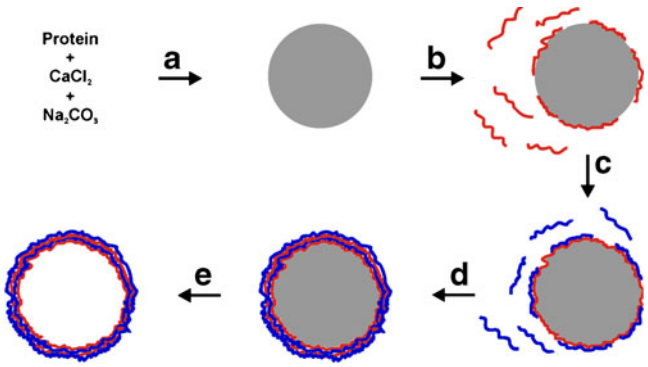


Fig. 1 Schematic representation of the preparation of protein-loaded polyelectrolyte microcapsules. During calcium carbonate core formation the protein is co-precipitated (**a**), followed by deposition of the first polyelectrolyte layer (**b**) and deposition of a second layer of an oppositively charged polyelectrolyte (**c**). Consecutive LbL coating with DEXS/pARG until the desired layer number is reached (**d**). Finally, hollow capsules with encapsulated protein are obtained by core dissolution with EDTA (**e**).

MATERIALS AND METHODS

Materials

Dextran sulfate (DEXS; Mw ~ 10 kDa), poly-L-arginine hydrochloride (pARG; Mw ~ 70 kDa), sodium chloride (NaCl), trehalose dihydrate, D-glucose, sucrose, hydrogen peroxide solution 30% (H_2O_2), Ampliflu Red and ovalbumin (OVA) were purchased from Sigma-Aldrich. Peroxidase from horseradish (HRP) of approximately 44 kDa was supplied as a freeze-dried powder with activity of 1000 units/mg (AZBTS as substrate) by Sigma-Aldrich. Calcium chloride ($CaCl_2$), sodium carbonate (Na_2CO_3), ethylenediaminetetraacetic acid (EDTA), di-sodium hydrogen phosphate anhydrous (Na_2HPO_4) and sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$) were all obtained from Merck. Phosphate-buffered saline (PBS) was purchased from Invitrogen. All water used in the experiments was of Milli-Q grade.

Preparation of Calcium Carbonate Microparticles

$CaCO_3$ particles were produced according to the following procedure (37). Equal volumes of 1 M solutions of $CaCl_2$

and Na_2CO_3 were thoroughly mixed using a magnetic stirrer. The precipitation reaction was allowed to proceed for 30 s at room temperature, after which the particles were centrifuged at 300 g for 3 min. The supernatant was removed, and the microparticles were resuspended in Milli-Q water. This washing step was repeated three times to remove unreacted products. Encapsulation of 500 μg HRP or 1 mg OVA was accomplished by co-precipitation with the $CaCO_3$ particles by mixing with the $CaCl_2$ solution prior to addition of the Na_2CO_3 solution.

Preparation of Polyelectrolyte Microcapsules

A schematic illustration of the preparation of (hollow) polyelectrolyte microcapsules is shown in Fig. 1. The first polyelectrolyte layer was deposited on the surface of the protein-loaded calcium carbonate particles by dispersing them in an aqueous polyelectrolyte solution containing 1 mg/ml dextran sulfate in 0.5 M NaCl. Adsorption was allowed to proceed for 10 min under continuous shaking. The excess of dextran sulfate was removed by three centrifugation (300 g, 3 min)/washing cycles. The following polyelectrolyte layer was deposited in the same way using a 1 mg/ml aqueous solution of oppositely charged polyelectrolyte, namely, poly-L-arginine in 0.5 M NaCl. This procedure was repeated until 2 or 3 bilayers were deposited. Finally, the calcium carbonate core was removed by adding 0.2 M EDTA, pH 6 (followed by washing steps), yielding hollow polyelectrolyte microcapsules. Finally, 125 μg HRP/ml microcapsules and 250 μg OVA/ml microcapsules were obtained.

Confocal Laser Scanning Microscopy (CLSM)

The structural integrity of the polyelectrolyte microcapsules before and after lyophilization was examined by confocal microscopy. Transmission images of polyelectrolyte microcapsules (re)suspended in water were obtained using a Nikon C1si confocal laser scanning module attached to a motorized Nikon TE2000-E inverted microscope (Nikon Benelux; Brussels, Belgium). Therefore, a drop of microcapsule suspension was placed on a cover glass and analyzed with CLSM using a water immersion objective lens (Plan Apo 60X, NA 1.2, collar rim correction, Nikon, Japan).

Scanning Electron Microscopy (SEM)

The morphological examination of the (lyophilized) microcapsules was performed by scanning electron microscopy (SEM). After lyophilization, microcapsules were redispersed in water, followed by one washing/centrifugation (300 g, 10 min) step. To that end, a drop of the microcapsule suspension was deposited onto a silicon wafer, air dried and then sputtered with a thin gold layer prior to SEM. The samples were analyzed with a Quanta 200 FEG FEI scanning electron microscope operated at 5 kV. SEM-EDX (energy-dispersive X-ray spectroscopy) analysis was also conducted to determine the chemical composition of the samples.

Lyophilization of Polyelectrolyte Microcapsules

Two hundred and fifty μl of a dispersion of polyelectrolyte microcapsules were centrifuged in eppendorf tubes at 300 g for 10 min, and the supernatant was discarded. Subsequently, 250 μl of various aqueous lyoprotectant solutions were added to the pellet; the resulting mixtures were vortexed and transferred to borosilicate lyophilization vials. Different polyols were tested as lyoprotectant in concentrations reported in literature, namely 2% (*w*/*v*) glucose, 5% (*w*/*v*) sucrose and 5% (*w*/*v*) trehalose.

The microcapsule dispersions in the vials were frozen and stored at −80°C prior to lyophilization, which was conducted in an Amsco-Finn Aqua GT4 freeze dryer (Finland). Vials were placed in the freeze dryer and lyophilized at a shelf temperature of −35°C and a chamber pressure of 0.8 mbar for 2 h. Then the shelf temperature was elevated to −15°C, and the pressure was kept at 0.8 mbar during the next 13 h. Subsequently, the temperature was increased to 10°C while the chamber pressure was reduced to 0.15 mbar for 9 h. Then samples were removed from the freeze dryer. Finally, lyophilized polyelectrolyte microcapsules were reconstituted in 250 μl of Milli-Q water or phosphate buffer by vortexing.

Enzymatic Activity of HRP

The enzymatic activity of HRP was determined by means of a fluorescence assay. The Ampliflu Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) was used as fluorogenic substrate which reacts with H_2O_2 (in a 1:1 stoichiometry) in the presence of HRP, producing highly fluorescent resorufin (excitation/emission maxima of 571/585 nm). The amount of HRP used for activity measurements was in the linear range found to be between 6,25 mU/ml and 0,2 mU/ml. Fluorescent measurements were taken with a Wallac Victor^2 Perkin Elmer fluorometer (535 nm excitation filter, 590 nm emission filter).

In brief, after washing and centrifugation steps, 50 μl aliquots of dilutions of reconstituted lyophilized polyelectrolyte microcapsules were transferred to a 96-well plate; subsequently, 50 μl of working solution (100 μM Ampliflu Red, 20 mM H_2O_2, 50 mM phosphate buffer pH 7.4) was added to the wells using a multichannel pipette, and reaction was allowed to proceed for 30 min before measurement of the fluorescence.

Antigen-Specific T-Cell Isolation

OVA-specific CD4+ and CD8+ T-cells were prepared from spleen and lymph node cell suspensions from OT-II and OT-I mice respectively. In brief, lymph nodes and spleens were crushed, and cell suspensions were pelleted. Erythrocytes were lysed using ammonium chloride lysis buffer, and the cells were passed through a cell strainer. CD4+ and CD8+ T-cells were isolated from the cell suspensions using Dynal mouse CD4/CD8 negative isolation kit (Invitrogen) according to the manufacturer's protocol.

T-Cell Proliferation Assay

Irradiated bone marrow-derived dendritic cells (BM-DCs) were pulsed with (lyophilized) OVA-loaded microcapsules in round-bottom 96-wells plates and washed three times. Purified T-cells, either CD4+ T-cells from OT-II mice or CD8+ T-cells from OT-I mice, were added to each well and co-cultured with antigen-pulsed BM-DCs for 48 h. After 48 h, [^{3}H]thymidine (1 μCi; Amersham Biosciences, NJ, USA) was added for 16 h to detect incorporation into DNA of proliferating T-cells. Cells were harvested onto filters, and [^{3}H]thymidine incorporation was assessed using a beta counter.

Immunization Protocols

Female C57BL/6 mice were purchased from Janvier (Le Genest Saint Isle, France) and housed in a specified pathogen-free facility. All mice were 8 to 10 weeks old at the onset of the experiments. All animals were treated according to the regulations of the Belgian law and the local Ethical Committee. Groups of four mice were immunized by subcutaneous injection of either 25 μg OVA encapsulated in polyelectrolyte microcapsules, 25 μg OVA encapsulated in microcapsules lyophilized in 5% (*w*/*v*) trehalose or 25 μg 'soluble OVA' mixed with empty microcapsules. Four weeks later, mice received a booster of the same formulation. Blood was collected by tail bleeding at weeks 7, 11, 14, 18 and 22 following the first immunization.

Determination of OVA-Specific Antibodies by ELISA

At different time points following immunization, blood samples were collected by tail bleeding, and serum was obtained after overnight incubation at 4°C. Ninety-six-well plates (Nunc) were coated overnight with ovalbumin (20 μg/ml) and incubated with serial dilutions of the serum samples. HRP coupled goat-anti-mouse-IgG_1 (Southern Biotechnology) was used as detection antibody for OVA-specific IgG_1. ELISA plates were developed using TMB substrate (BD Pharmingen). After the reaction was stopped with 1 M H_2SO_4 (Sigma-Aldrich), optical density (O.D) was measured at 450 nm using an Envision plate reader (Perkin Elmer). Antibody titers were determined as the dilution at which the O.D. reaches a value of three times the O.D. of the blank (mean ± standard deviation values, $n=4$).

Statistical Analysis

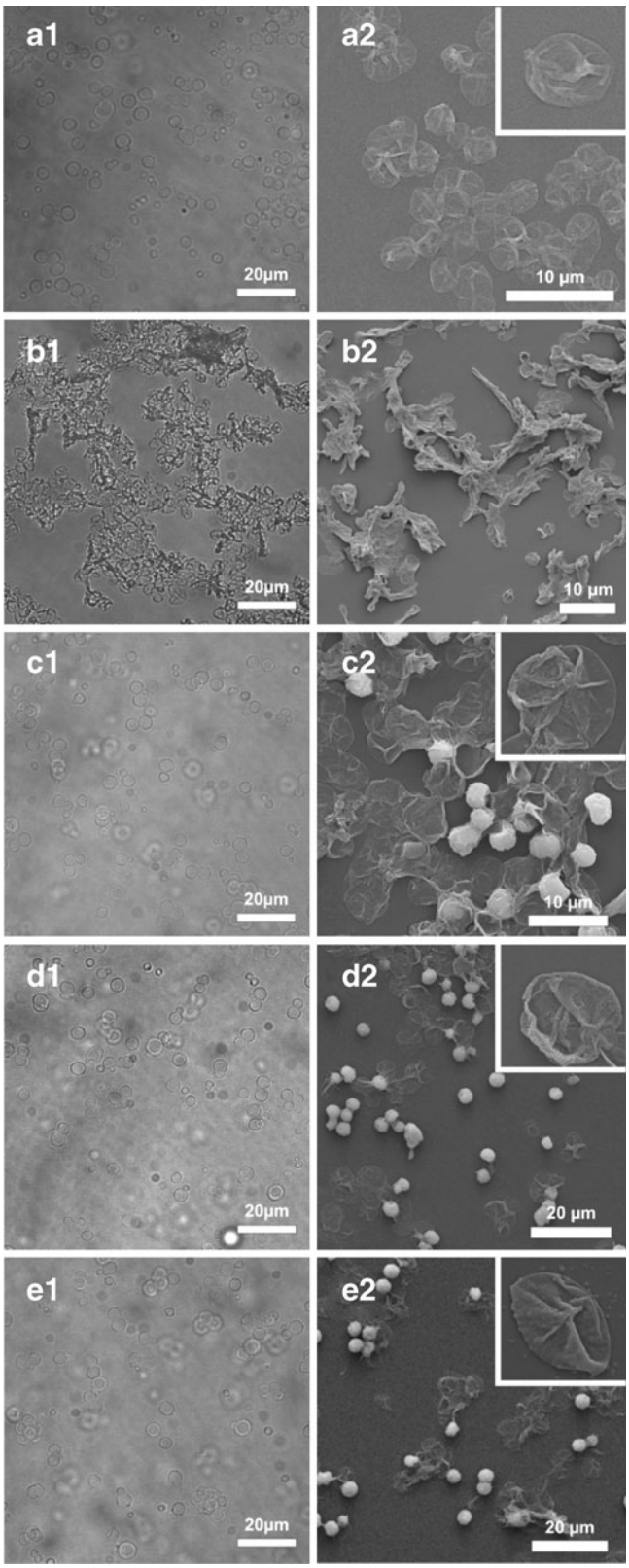
For *in vitro* assays, data represent the mean ± standard deviation of experiments performed in triplicate. For *in vivo* evaluation, antibody titers are expressed as mean ± standard deviation values ($n=4$). Comparisons were made by *t* test. Statistical significance of differences between free OVA and OVA-loaded polyelectrolyte microcapsules before and after lyophilization was evaluated using a Student's *t* test. A value of $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Lyophilization of Polyelectrolyte Microcapsules

$CaCO_3$ microparticles were used as sacrificial templates for the fabrication of (hollow) polyelectrolyte microcapsules. Dextran sulfate (DEXS) and poly-L-arginine (pARG) were selected as negatively and positively charged polyelectrolytes, respectively, for LbL assembly. Figure 1 schematically shows the procedure of microcapsule fabrication. In the first step, calcium chloride and sodium carbonate are mixed in the presence of the protein to be encapsulated, leading to the formation of calcium carbonate microparticles containing the protein in its pores. In the next step, three bilayers of DEXS/pARG were deposited on the $CaCO_3$ microparticles, and, finally, the $CaCO_3$ microparticles were dissolved by treatment with an aqueous EDTA solution. Figure 2a1 and a2 show the morphology of the thus obtained polyelectrolyte microcapsules, as obtained by CLSM and SEM.

The polyelectrolyte microcapsules were lyophilized from an aqueous dispersion. Following lyophilization, the solid residue was redispersed in water, and the morphology of the microcapsules was studied by CSLM (Fig. 2b1) and SEM (Fig. 2b2). Extensive aggregation of the microcapsules was observed, and a proper redispersion of the microcapsules in water appeared to be impossible. To cope with these issues, different polyols, such as glucose, sucrose and trehalose, which are widely applied as stabilizers during freeze-drying (38,39), were evaluated as lyoprotectants.



◀ **Fig. 2** Transmission microscopy images of $(DEXS/pARG)_3$ microcapsules respectively prior to lyophilization (**a1**), upon reconstitution after lyophilization in the absence of lyoprotectant (**b1**) or in the presence of glucose (**c1**), sucrose (**d1**) and trehalose (**e1**). Scanning Electron Microscopy images of microcapsules respectively before (**a2**) and after lyophilization in the absence of lyoprotectant (**b2**) or in the presence of glucose (**c2**), sucrose (**d2**) and trehalose (**e2**). In the right corner, insets show enlarged images of individual microcapsules.

Figures 2(c1, d1, e1) and (c2, d2, e2) show confocal and electron microscopy images of capsules lyophilized in the presence of the different lyoprotectants. Contrary to lyophilization in 'pure' water, addition of any of the polyols prevented cracking of the capsules' shells and aggregation of the polyelectrolyte microcapsules. Moreover, the microcapsules could be easily redispersed in water with preserved integrity. Owing to sample preparation, the polyols crystallized out during drying of the sample; sugar crystals could be readily recognized on the SEM images as white structures (Fig. 2c2–e2). By SEM-EDX analysis we could exclude the presence of $CaCO_3$ inside the microcapsules and confirm that the crystals are formed by residual polyols (data not shown).

The destruction of the polyelectrolyte microcapsules upon lyophillization in pure water is due to the presence of water both in and around the microcapsules. During the freezing step in the lyophillization cycle, the growing, sharp ice crystals may exert a mechanical stress on the capsules and may pierce their shell, eventually leading to the rupture of the microcapsule wall. In contrast, in the presence of lyoprotectants, an amorph, glassy matrix is formed in which the water is dissolved and crystallization of water is suppressed, thereby (a) limiting mechanical damage of the polyelectrolyte microcapules and (b) preventing microcapsules from sticking together.

Lyophilization of HRP-Loaded Polyelectrolyte Microcapsules

It has been reported that enzymes retain their activity upon encapsulation inside polyelectrolyte microcapsules (28–33,37). Therefore, we evaluated whether the encapsulated enzymes survive lyophilization. HRP was encapsulated in polyelectrolyte microcapsules through co-precipitation with calcium carbonate; the $CaCO_3$ core templates were coated with 3 bilayers DEXS/pARG and subsequently dissolved. The obtained HRP-loaded $(DEXS/pARG)_3$ microcapsules were lyophilized in aqueous medium containing glucose, sucrose or trehalose. Next, the solid residue was redispersed in phosphate buffer, and the activity of HRP was quantified by a fluorescent assay. The low molecular weight substrate ampliflu Red diffuses through the polyelectrolyte membrane; once inside the

polyelectrolyte microcapsules it can be oxidized by the encapsulated HRP to the highly fluorescent resorufin which diffuses out of the microcapsule.

Figure 3 displays the remaining enzymatic activity after lyophilization of HRP-loaded microcapsules. Lyophilization of the microcapsules in the absence of polyols led to a 90% reduction of the HRP activity. However, when the polyelectrolyte microcapsules were lyophilized in the presence of any of the tested polyols, over 70% of the HRP activity was retained. When lyophilization of HRP-loaded microcapsules was performed in a polyol containing sodium phosphate buffer, a major fraction of the HRP activity was lost (data not shown). In this buffer, the dibasic salt $Na_2HPO_4.H_2O$ is less soluble than the monobasic salt, leading to its precipitation during freezing. This selective buffer crystallization, known as the freeze-concentration effect (40), causes a decrease in pH (≈3 pH units) which can have a detrimental impact on proteins sensitive to pH shifts.

Our study demonstrates that the presence of polyols in the formulation enhances protein stability and preserves the native conformation of the encapsulated enzyme. Usually, polyols, such as sucrose and trehalose, are employed as stabilizers to minimize protein denaturation during processing and storage (41,42). Two different hypotheses are proposed to elucidate the stabilization mechanisms performed by polyols. The first one is the "water substitute" hypothesis; this theory states that polyols can form hydrogen bonds at specific sites at the surface of the protein. They act as "water substitutes" and replace the water that is lost during the drying process resulting in preservation of the native protein conformation (43). This is a thermodynamic approach to explain the stabilization mechanism of polyols. The second one, the glass dynamics hypothesis, formerly called the vitrification hypothesis, considers a purely kinetic stabilization mechanism. During freezing the polyols form a glassy matrix characterized by a high viscosity. In this rigid matrix, the proteins become less mobile, minimizing molecular interactions and slowing down degradation reactions (44).

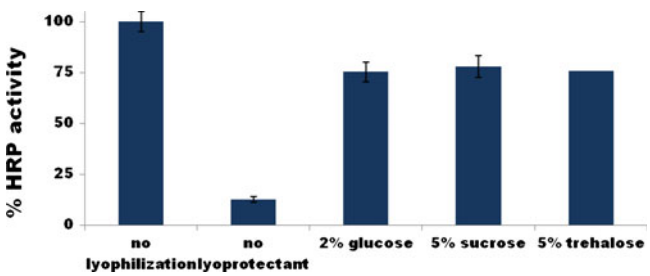


Fig. 3 The enzymatic activity of HRP after redispersion of lyophilized HRP-loaded $(DEXS/ARG)_3$ microcapsules in phosphate buffer. HRP activity prior to lyophilization of the microcapsules was set as 100%.

In Vitro T-Cell Proliferation Assay of Lyophilized OVA-Loaded Polyelectrolyte Microcapsules

As our final aim is to develop a dry vaccine formulation of antigen-loaded polyelectrolyte microcapsules, we evaluated the effect of lyophilization on the ability of antigen-loaded polyelectrolyte microcapsules to induce antigen presentation by dendritic cells to T-cells in an *in vitro* setting. Therefore, OVA was encapsulated as a model antigen in $(DEXS/pARG)_2$ polyelectrolyte microcapsules. After phagocytosis by DCs, the polyelectrolyte microcapsules can be degraded by proteolytic enzymes in the phagosome, resulting in release of encapsulated antigen (34). The activity of the OVA-loaded microcapsules before and after lyophilization in the presence of trehalose was evaluated by an *in vitro* T-cell proliferation assay. Different amounts of soluble OVA or OVA-loaded microcapsules were used to pulse DCs. Subsequently, pulsed DCs were co-cultured with OVA-specific CD4+ OT-II or CD8+ OT-I cells and proliferation of T-cells was assessed by $[^3H]$-thymidine incorporation.

As seen in Fig. 4a, encapsulation of OVA in polyelectrolyte microcapsules led to an enhanced MHC class II presentation and CD4+ T-cell proliferation, compared to soluble OVA, especially at lower OVA concentrations. Statistical analysis revealed significant differences ($p<0.05$) between soluble and encapsulated OVA. The results indicate that encapsulation of OVA in polyelectrolyte microcapsules

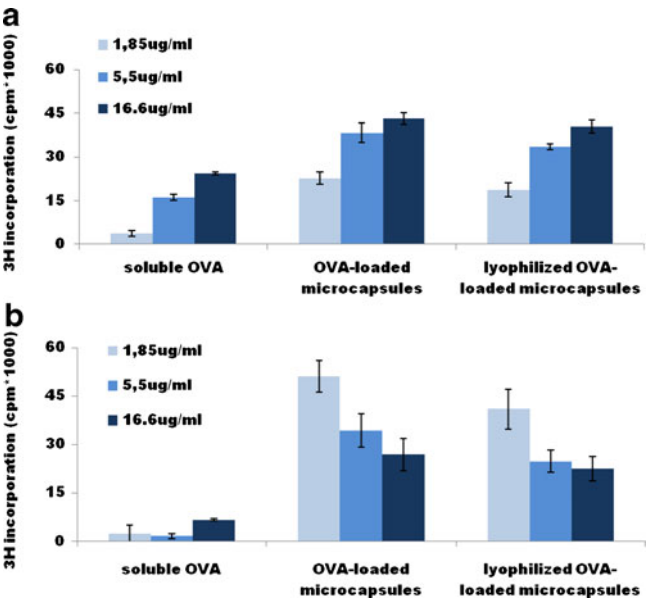


Fig. 4 (**a**) Proliferation of OVA-specific *OT-II T-cells* after OVA presentation by DCs. DCs were first pulsed with soluble OVA, non-lyophilized OVA-loaded microcapsules or lyophilized OVA-loaded microcapsules. (**b**) Proliferation of OVA specific *OT-I T cells* after OVA presentation by DCs. DCs were first pulsed with soluble OVA, non-lyophilized OVA-loaded microcapsules or lyophilized OVA-loaded microcapsules.

enhances antigen presentation by DCs and subsequent proliferation of T-cells *in vitro* compared to soluble OVA. Note that the lyophilized OVA-loaded polyelectrolyte capsules performed as effectively as the non-lyophilized ones (the differences were not significant).

Figure 4b shows that, compared to soluble OVA, encapsulation of OVA also resulted in an enhanced MHC class I antigen cross-presentation to OT-I cells and CD8+ T-cell proliferation ($p<0.05$). Similarly to OT-II proliferation, no statistically significant difference in T-cell activation was observed between the lyophilized and the non-lyophilized microcapsules.

Immunization Performance of Lyophilized OVA-Loaded Polyelectrolyte Microcapsules

As a proof-of-principle, we compared the *in vivo* immunization potential of lyophilized and non-lyophilized OVA-loaded microcapsules; groups of C57BL/6 mice were immunized by a subcutaneous injection of 25 μg of OVA encapsulated in $(DEXS/pARG)_2$ microcapsules. Empty microcapsules mixed with an equivalent dose of soluble OVA served as control. Animals were boosted 4 weeks later with the same formulation. The levels of specific anti-OVA IgG_1 antibody in serum were determined following the second immunization at 3–4 week intervals over a period of 22 weeks.

As depicted in Fig. 5, the antibody levels obtained in mice immunized with lyophilized and non-lyophilized formulations of OVA encapsulated in microcapsules were significantly higher ($p<0.05$) than those measured in mice which were injected with a mix of empty microcapsules and soluble OVA. These results confirm the lack of immunogenicity of soluble OVA and indicate that encapsulation of antigen in particulate formulations enhances the immune response. Furthermore, it is clear that non-lyophilized OVA-loaded microcapsules elicited much higher antibody titers than the lyophilized ones, suggesting that the lyophilization process has an impact on the immunization potency of the OVA-loaded microcapsules. Note, however, that the antibody titers in mice immunized with lyophilized OVA-loaded microcapsules were still significantly higher than those in the control group. Based on the outcome of our experiments, we strongly believe that a further optimization of the lyophilization of OVA-loaded microcapsules is possible (e.g., using another lyophilization cycle, other lyoprotectants), which might result in still better performing OVA-loaded microcapsules.

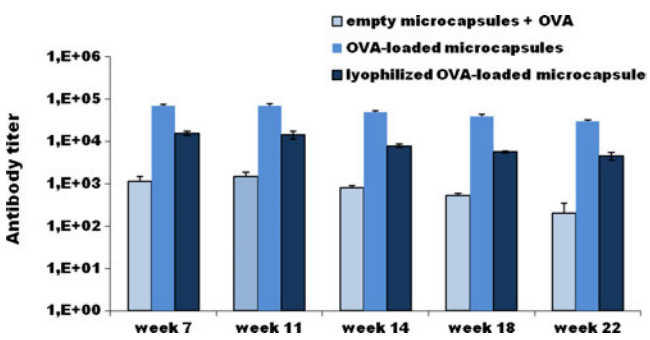


Fig. 5 Serum anti-OVA IgG_1 levels following subcutaneous administration of OVA-loaded microcapsules, lyophilized OVA-loaded microcapsules or empty microcapsules mixed with free OVA. Boosters of the same formulation were administered at 4 weeks.

CONCLUSIONS

Polyelectrolyte microcapsules fabricated by LbL assembly show great potential as drug delivery vehicles for antigen delivery. However, stability of the proteins encapsulated in the aqueous lumen of the polyelectrolyte microcapsules might limit the application of polyelectrolyte microcapsules as protein carriers. We showed that, in absence of a lyoprotectant, polyelectrolyte capsules do not survive a lyophilization process; capsules aggregate and become ruptured, while encapsulated HRP loses its activity. However, when a lyoprotectant was used, intact microcapsules were found upon reconstitution of the lyophilized microcapsules, while HRP retained up to 70% of its original activity. Our observations clearly show that polyols as lyoprotectants are well suited to keep both the polyelectrolyte microcapsules and the encapsulated proteins stable during freeze-drying processes.

Finally, we assessed the immunogenicity of lyophilized and non-lyophilized antigen-loaded microcapsules in an *in vitro* T-cell proliferation assay. Our data indicate that encapsulation of OVA in polyelectrolyte microcapsules leads to an enhanced antigen presentation and amplification of the T-cell proliferation compared to soluble OVA. We also demonstrated that lyophilized OVA-loaded polyelectrolyte microcapsules did not lose immunological activity as compared to non-lyophilized microcapsules in an *in vitro* T-cell proliferation assay. These observations reveal lyophilization as a suitable strategy to obtain a dry formulation of antigen-loaded polyelectrolyte microcapsules. However, *in vivo* experiments revealed a lower immunogenicity of lyophilized OVA-loaded microcapsules. Optimization of the lyophilization procedure might overcome this hurdle. Moreover, it was shown that polyelectrolyte microcapsules are an excellent adjuvant for antigen delivery, since both *in vitro* T-cell proliferation as well as *in vivo* antibody production show much stronger immune activation than free non-encapsulated OVA. These findings point out that polyelectrolyte microcapsules are promising carriers for antigen delivery not only because of improved antigen presentation but also because a stable, dry formulation can be obtained.

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