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Hydroxyethylstarch microcapsules: A preliminary study for tumor immunotherapy application

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Abstract

The objective of this work was to prepare microcapsules which would allow protection and slow release of antigens used for melanoma immunotherapy treatment. Hydroxyethylstarch (HES) microcapsules were prepared using interfacial cross-linking with terephthaloyl chloride (TC). They were characterized with respect to morphology (microscopy) and size (in the 4–15 μm range). Bovine serum albumin (BSA) was used as model protein for loading and release studies. Microcapsules were loaded with solutions at different protein concentrations (0.5–5%). The maximum loading efficiency (20%) was observed with the concentration of 2.5%, which allowed a loading capacity near 100%. Confocal laser scanning microscopy (CLSM) visualization showed that BSA was entrapped within the microcapsules and not only associated to their outer surface. BSA-release studies showed a 20% BSA release within 30 min while 80% remained entrapped in the microcapsules for 4 days. Microcapsules were degraded by α-amylase and addition of esterase to α-amylase enhanced slightly their degradation. In vitro studies on melanoma cells showed that HES microcapsules were non-toxic. Preliminary in vivo studies demonstrated that microcapsules were biodegradable after intraperitoneal injection (i.p.). The observation of peritoneal wash showed a complete degradation within 7 days, indicating a possible application as an in vivo drug delivery system especially to enhance the presentation of antigens.

Keywords: Hydroxyethylstarch; Biodegradable microcapsules; Confocal microscopy; Loading efficiency; Cytotoxicity; Enzymatic degradation

1. Introduction

The relative resistance of melanoma cells to chemotherapy has led to search for alternative treatment options, including immunotherapy (Komenaka et al., 2004). This technique involves the treatment of cancer through manipulation of the immune system (Pardoll, 1998; Nawrocki and Mackiewicz, 1999; Vasey, 2000; Hoffman et al., 2000; Jager et al., 2001). Such an approach needs an appropriate presentation of tumor-specific antigens (Thumann et al., 2003). The use of biodegradable, polymeric systems for tumor immunotherapy has received limited consideration as compared to more conventional cell-based approaches (Golumbek et al., 1993; Egilmez et al., 2000; Kuriakose et al., 2000; Denis-Mize et al., 2000). Microparticulate antigen delivery systems are of special interest as stable carriers and effective adjuvants for the delivery of vaccines (Thomasin et al., 1996; Raychaudhuri and Rock, 1998; Strindelius et al., 2002). Attraction is based on different advantages, such as the enhancement of drug stability and the controlled drug release (Pappo et al., 1991; Eldridge et al., 1991; Degling and Stjärnkvist, 1995; Men et al., 1996; Putney and Burke, 1998). We focused our attention on the development of such devices to enhance the presentation of antigens in immunotherapy. Starch-based microparticles are suitable carriers for protein delivery systems due to their biocompatibility, shelf-life stability, high loading capacity, biodegradability, and controlled release of the encapsulated drug (Laakso et al., 1987; Heritage et al., 1996; Larionova et al., 1999; Sturesson and Wikingsson, 2000). Native starch may not be appropriate to prepare parenteral controlled drug delivery systems, since it is rapidly degraded in vivo and many drugs are released too quickly from such unmodified starch systems (Henrist et al., 1999; Pereswetoff-Morath, 1998; Michailova et al., 2001). In contrast, hydroxyethylstarch (HES) is a less quickly degraded

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starch derivative, widely used for therapy and prophylaxis of all kinds of volume deficiencies. Because of its major attractions including biocompatibility and biodegradation properties, HES was chosen for the production of microcapsules intended to further vectorization in vivo. Moreover, we have previously shown that HES leads to stable microparticles by interfacial cross-linking (Levy and Andry, 1990). These HES microparticles had never been used for the encapsulation of large molecules like proteins and they had never been tested in vivo. So different studies were realised to characterize these particles. The morphology and the size of HES microparticles were evaluated using light microscopy, confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM) and laser diffraction granulometry. Loading and release characteristics were investigated with a model protein: bovine serum albumin (BSA). Degradation was evaluated in vitro in presence of amylase and esterase. Furthermore, cytotoxicity studies on melanoma cell line, and in vivo biodegradation were evaluated.

2. Materials and methods

2.1. Materials

Hydroxyethylstarch (Voluven®) was purchased from Fresenius Kabi (Sèvres, France) and terephthaloyl chloride from Acros Organics. Esterase (19 IU/mg) from porcine liver, α-amylase (19.5 IU/mg) from porcine pancreas and bovine serum albumin were from Sigma (St. Quentin Fallavier, France). Chloroform, cyclohexane and ethanol, were of analytical grade and provided by SDS (Peypin, France). Texas red labeled BSA was purchased from Invitrogen (Cergy–Pontoise, France). Surfactants were polysorbate (Tween® 20) and sorbitan trioleate (Span® 85) from Seppic (Paris, France). Methylene Blue and fluoresceinamine were from Fluka (St. Quentin Fallavier, France).

2.2. Preparation of HES microparticles

Microcapsules were prepared by the interfacial cross-linking method according to our protocol (Levy and Andry, 1990). Briefly, a 20% (w/v) HES solution was prepared in carbonate buffer pH 9.8. This aqueous phase (6 ml) was emulsified under mechanical agitation (5000 rpm) in cyclohexane (30 ml) containing 5% (v/v) Span® 85. After 5 min, 40 ml of a 5% (w/v) solution of terephthaloyl chloride in chloroform/cyclohexane (1/4, v/v) were added to the emulsion and stirring was prolonged for 30 min. The reaction was stopped by dilution with 40 ml of chloroform/cyclohexane (1/4, v/v). Then, microparticles were washed with cyclohexane (4×), with ethanol 95% (v/v) containing 2% (v/v) Tween® 20 (1×), then with ethanol 95%(v/v) (2×) and with water (4×). Finally, microcapsules were re-suspended in water and lyophilized.

2.3. Microparticles characterization

2.3.1. Morphology and size

Light microscopy (Olympus BH-2, Olympus, Shibuya-Ku, Tokyo, Japan) was performed for initial visualization of the HES microcapsules. Further morphology studies were carried out by means of confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). For CLSM study, fluorescent microparticles were prepared by incorporating 30 mg of fluoresceinamine in the HES solution before the emulsification step. A Bio-Rad MRC 1024 system (Bio-Rad, Hercules, CA, USA) mounted on Olympus IX70 Axioplan optical microscope (Olympus, Shibuya-Ku, Tokyo, Japan) was used. All acquisitions were made using UPlan FI×63, 1.4 numerical aperture objective. Acquisitions were performed by exciting fluoresceinamine with the 488-nm line of an air-cooled argon ion laser. Eighty sections per microparticle were recorded with a 0.30-μm *z*-step to collect the whole volume with a sufficient *z*-sampling. Files were then transferred to a Sun Sparc 20 workstation (Sun Microsystems, Mountain View, CA, USA) for further processing.

For SEM, lyophilized microparticles were deposited on double-faced adhesive and coated with palladium/gold before observation. Samples were observed under a scanning electron microscope (JEOL 5400 LV) (JEOL, Schiphol, The Netherlands) at 15 kV to study the shape and surface morphology.

The samples for Fourrier transform-infrared (FT-IR) study were prepared according to the standard technique: 1 mg of lyophilized microcapsules or HES was ground with 190 mg of kBr. The mixture was compressed in tablets, 1 mm thick, under a pressure of 10 kPa. FT-IR spectra were obtained from a Perkin-Elmer Spectrum BXII spectrometer (Perkin-Elmer, Courtaboeuf, France).

Particles were sized by a laser diffraction technique (Coulter Particle Sizer, type LS 200, Coultronics, France). Size distribution was displayed in terms of volume versus particle size.

2.3.2. Enzymatic degradation of HES microparticles

Lyophilized microcapsules (10 mg) were suspended in 5 ml phosphate buffer (20 mM sodium phosphate, 6 mM NaCl, pH 7) containing either α-amylase (19.5 IU/ml), esterase (19 IU/ml) or a mixture of both enzymes. The samples were incubated under shaking at 37 °C. The microparticle morphology was observed by light microscopy and the concentration was evaluated on Malassez cell in aliquots withdrawn at appropriate time intervals.

2.3.3. Bovine serum albumin loading

The BSA loading of microparticles was obtained by incubating 5 mg of HES microparticles in 1 ml of a 0.5–5% (w/v) BSA solution in phosphate buffer saline (PBS) pH 7.4. Tubes were incubated 3 h under shaking at 37 °C. Then, they were centrifuged (1400 rpm for 30 min) to remove the unloaded BSA. The unloaded BSA in the supernatant was quantified with the Bradford protein assay method (Bradford, 1976). Loading efficiency (LE) was determined as: LE = [(total amount of BSA)−(unloaded BSA)]/total amount of BSA (Van der Lubben et al., 2001).

CLSM was used to demonstrate effective BSA loading. Microparticles were incubated with a 2.5% Texas red labeled-BSA solution (Molecular Probes, Invitrogen) as afore-

mentioned. Acquisitions were performed by exciting with the 568 nm line of an air-cooled krypton/argon ion laser.

2.3.4. Bovine serum albumin release

For BSA release, 5 mg of microparticles were loaded as previously described. After centrifugation, loaded microcapsules were re-suspended in PBS pH 7.4 to make a 1% (w/v) microparticles suspension. Samples were incubated under gentle shaking at 37 °C during 30, 60, 90, 120, 180 and 240 min, 24 and 48 h and 4 days (one tube for each time). The tubes were given a spin-off and unbound BSA present in the supernatant was determined with the Bradford protein assay method (Bradford, 1976).

2.4. Cell and culture conditions

A murine melanoma cell subline, denoted B16-R, resistant to 3.5×10^{-7} M doxorubicin, derived from the ATCC stock was isolated at the National Tumor Institute in Milan (Mariani and Supino, 1990) by stepwise selection in increasing concentrations of doxorubicin. B16-R cells were grown in a 5% CO_2 atmosphere at 37 °C, in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen). To generate spheroids, exponentially monolayer growing B16-R cells were detached by trypsinization and 100 μl culture medium containing 50×10^3 cells were added to each well of a 96-well microplate, previously coated with 40 μl 1.33% agarose (Sigma). Microplates were placed on a 3-D stirring machine (Polymax 1040, Heidolph, Germany) for overnight incubation. After 3 days at 37 °C, 5% CO_2, the medium was changed and the cytotoxicity assays could be initiated.

2.5. Microparticle cytotoxicity

A MTT (Sigma) colorimetric assay was performed. Cell viability was determined by measuring the optical density differences between 550 and 650 nm using the 550 microplate reader model (Bio-rad, Marnes la Coquette, France). The cell surviving fraction was determined by dividing the mean absorbance values of treated samples by the mean absorbance of untreated control samples.

2.6. Animals

Female B6D2F1 mice (6-week-old) were obtained from Charles River Laboratories (Iffa Credo, L'Arbresle, France) and housed at the maintenance facility of the School of Pharmacy of Reims. All experiments were carried out in compliance with the regulations of the Animal Care and Use at the School of Pharmacy of Reims.

2.7. In vivo biodegradation studies

Blank lyophilized microparticles (5 mg) were re-suspended in 200 μl of sterile physiological saline (a 0.9% NaCl (w/v) aqueous solution) and incubated at 37 °C with permanent shaking until adequate re-swelling. Then the microparticle suspension was administered by intraperitoneal injection (i.p.) on a group of 21 female B6D2F1 mice. Daily, three mice were sacrificed, the intraperitoneal cavity was washed twice with 10 ml of physiological saline and the microparticle degradation was assessed by light microscopy.

3. Results and discussion

3.1. Morphology and size of microparticles

The morphology of the microparticles prepared from a 20% (w/v) HES solution with 5% (w/v) terephthaloyl chloride concentration was analyzed by light microscopy after suspension in water and staining with Methylene Blue. They appeared transparent, spherical and well individualised (Fig. 1a). The size of these HES microparticles was found to range from 4 to 15 μm with an average of 8.3 ± 1.9 μm. Microcapsule lyophilization gave white and free-flowing powders. Moreower, the particles were intact and easily recovered their spherical shape after rehydratation in aqueous solutions. These particles exhibited a continuous and smooth surface, as shown by scanning electron microscopy (Fig. 1b). Three-dimensional reconstruction of the microparticles, using CLSM, allowed us to visualize a cross-section and perfectly illustrated that particles were hollow spheres with a concave membrane (Fig. 2a).

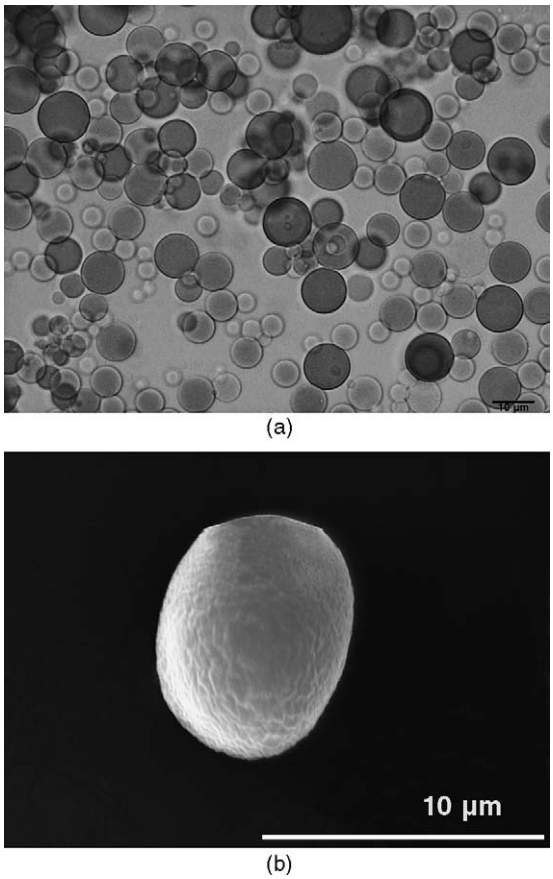
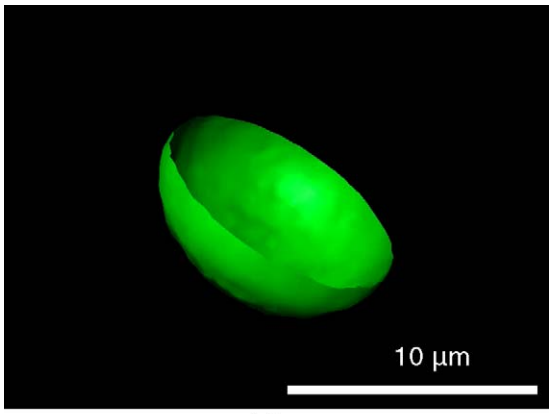
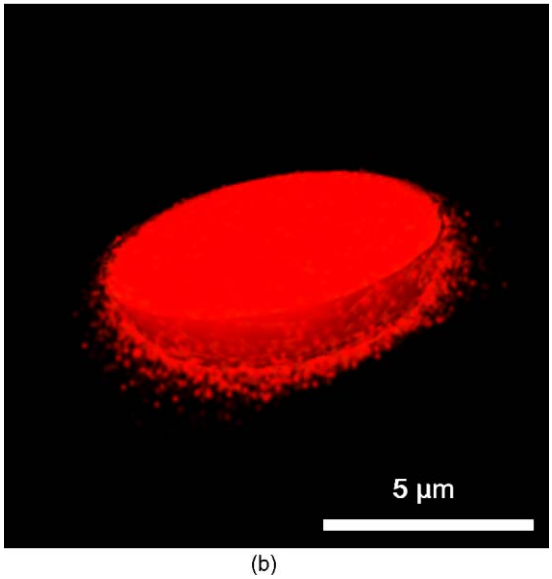


Fig. 1. HES microparticles: (a) optical photomicrograph (scale bar = 10 μm) and (b) SEM (scale bar = 10 μm).



(a)



(b)

Fig. 2. Three-dimension reconstruction of the maximum projection from *z*-series ($n = 80$) of confocal fluorescent image of optical section of single microcapsule: (a) labeled with fluoresceinamin; scale bar = 10 μm and (b) loaded with BSA-Texas red (2.5%); scale bar = 5 μm (all sections were recorded using magnification ×63 and zoom 3×).

The IR spectra of the microcapsules were compared with the spectrum of original HES in Fig. 3. As it was expected with our previous studies (Andry et al., 1998), the main modifications on microcapsules spectra were two bands at 1717 and 1277 cm^{-1}, which reflected the formation of esters bonds from hydroxy groups of HES.

3.2. In vitro degradation of HES microparticles

Degradation by various enzyme solutions was studied: esterase, α-amylase or both enzymes. Esterase usually allows the disruption of ester bonds involved in the microparticles wall formation and α-amylase is the enzyme which degrades starch and derivatives like HES. The enzyme concentration used in these assays were the same that allowed the degradation of particles prepared by cross-linking of soluble starch with terephthaloyl chloride (Larionova et al., 1999). We observed that the microcapsules were resistant to digestion by the esterase solution (19 IU/ml). However, α-amylase (19.5 IU/ml) exhibited a progressive degradation activity: 10% of microcapsules were degraded after 2 h and 50% after 24 h. Addition of esterase (19 IU/ml) to α-amylase enhanced slightly the rate of microcapsules degradation; after incubation times of 6 and 24 h, the degradation of microcapsules (%) were, respectively, 40% versus 50% and 50% versus 70% with amylase alone versus both enzymes (Table 1). The same observation was performed for microcapsules prepared with soluble starch (Larionova et al., 1999). This phenomenon is to link with the esterase activity which is only possible after partial degradation of HES by amylase.

3.3. Bovine serum albumin loading efficiencies of HES microparticles

Five milligram of HES microcapsules were suspended in 1 ml of BSA solution in PBS pH 7.4 (5 mg will be the quantity of microcapsules used for further in vivo tests). Loading efficiency was determined with 0.5–5% (w/v) BSA solutions. Fig. 4 shows that the LE presented a maximum value for the concentration of 2.5% (w/v). The obtained loading efficiency was $19.64 \pm 0.48\%$ ($n = 5$) for independently prepared batches. Therefore, 2.5% (w/v) BSA in the loading solution was selected as the optimal concentration. Under these conditions, the loading capacity value (LC = [loaded BSA/weight microcapsules] × 100) was $98.2 \pm 2.4\%$. With CLSM studies, it was possible to visualize both the inside and the surface of the Texas red labeled particles. Results obtained with this technique showed that BSA was not only associated to the surface, but also entered within the HES microcapsules (Fig. 2b). The repartition of BSA was homogeneous in the microcapsule and did not present aggregates. These particles, with a thin wall allowed the encapsulation of an important amount of protein in their cavity. While microspheres in the same range of size allow a smaller albumin loading, near 40% (Van der Lubben et al., 2001). This high loading capacity is a good point for further uses since large amount of protein was encapsulated in a minimal amount of polymer. This reduces the mass of the material to be administered (Sinha and Thehan, 2003). Moreover, HES microcapsules loading does not require a coupling reaction as it has to be done with polyacryl starch microparticles (Degling and Stjärnkvist, 1995).

3.4. BSA release from HES microparticles

BSA release from HES microparticles re-suspended in PBS pH 7.4 was determined after 30, 60, 90, 120, 180 and 240 min, 24 and 48 h and 4 days. After an initial release of 20% during the first 30 min, no BSA was released for the following 4 days. These results indicated that 80% of the BSA remained entrapped in the microcapsules under these conditions. These results are to be correlated with those obtained by Van der Lubben et al. (2001); chitosan microparticles (in the same range of size) loaded with ovalbumin allowed a release of about 10% of the loaded protein. So, an important part of albumin will only be released after complete degradation of the HES microcapsules since they are degradable. The protein is likely to be protected within the particle and this would allow a progressive release in vivo.

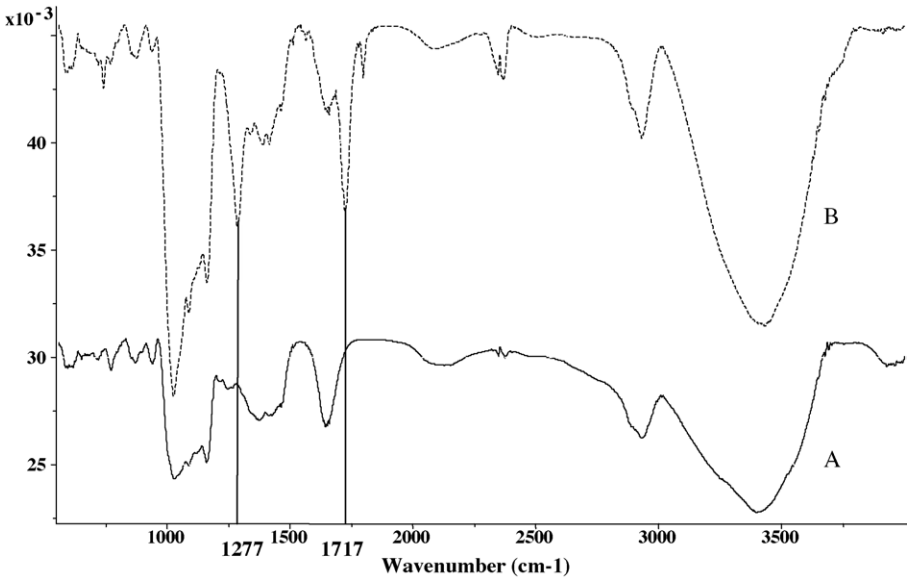


Fig. 3. FT-IR spectra: (A) original HES and (B) hydroxyethylstarch microcapsules.

Table 1
Degradation of microcapsules in presence or absence of enzymes as a function of enzymatic incubation time; percentage of degraded microcapsules counted on a Malassez cell

Incubation time (h)	Without enzymes (%)	Amylase (19.5 IU/ml) (%)	Esterase (19 IU/ml) (%)	Amylase + Esterase (19.5 IU/ml + 19 IU/ml) (%)
2	–	10 ± 2	–	10 ± 2
4	–	35 ± 3	–	35 ± 3
6	–	40 ± 4	–	50 ± 4
24	–	50 ± 5	–	70 ± 5

Results are the mean of three determinations ± S.D.

3.5. In vitro studies of HES microcapsules cytotoxicity on B16-R melanoma cells

The microcapsules cytotoxicity was tested on the B16-R melanoma cell line, resistant to doxorubicin, cultivated in monolayers or as tri-dimensional models. One milliliter of microcapsule suspension was added in each well of a 96-well microplate, each filled with 100 μl of suspension containing 50×10^3 cells. Results (Table 2) show that microcapsules, in the concentration range of 0.1–1 mg/ml, did not exhibit a significant cytotoxicity against this cell line after 72 h (all values <10%, $n = 9$).

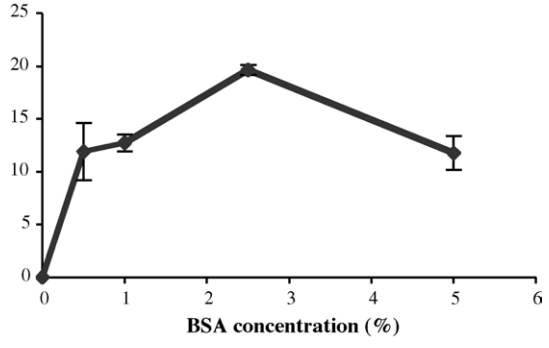


Fig. 4. The influence of BSA initial concentration on loading efficiency of HES microcapsules. Data are expressed as mean ± S.D. of five experiments.

3.6. In vivo biodegradation studies

Administration by intraperitoneal route was used because in our future experiments, microcapsules loaded with antigens will be injected by this route since it was shown that it is a particularly effective route for stimulating immune responses (Degling and Stjärnkvist, 1995; Cleland, 1999).

In a preliminary assay, HES microcapsules (5 mg) were resuspended in 200 μl of sterile physiological saline and injected intraperitoneally in two groups of three female B6D2F1 mice.

Table 2
Growth inhibition of B16-R melanoma cells cultured in monolayers or in spheroids (50×10^3 cells/100 μl), incubated with different concentrations of microparticles (mg/ml) after a 72 h incubation time

	Microcapsules (mg/ml)					
	0.1	0.2	0.3	0.4	0.8	1
Growth inhibition (%)						
B16-R monolayers	0.5 ± 1	1 ± 1	3 ± 1	5 ± 1	7 ± 1	8 ± 1
B16-R spheroids	0	0	3 ± 1	5 ± 1	6 ± 1	7 ± 1

Results are given as means ± S.D. ($n = 9$).

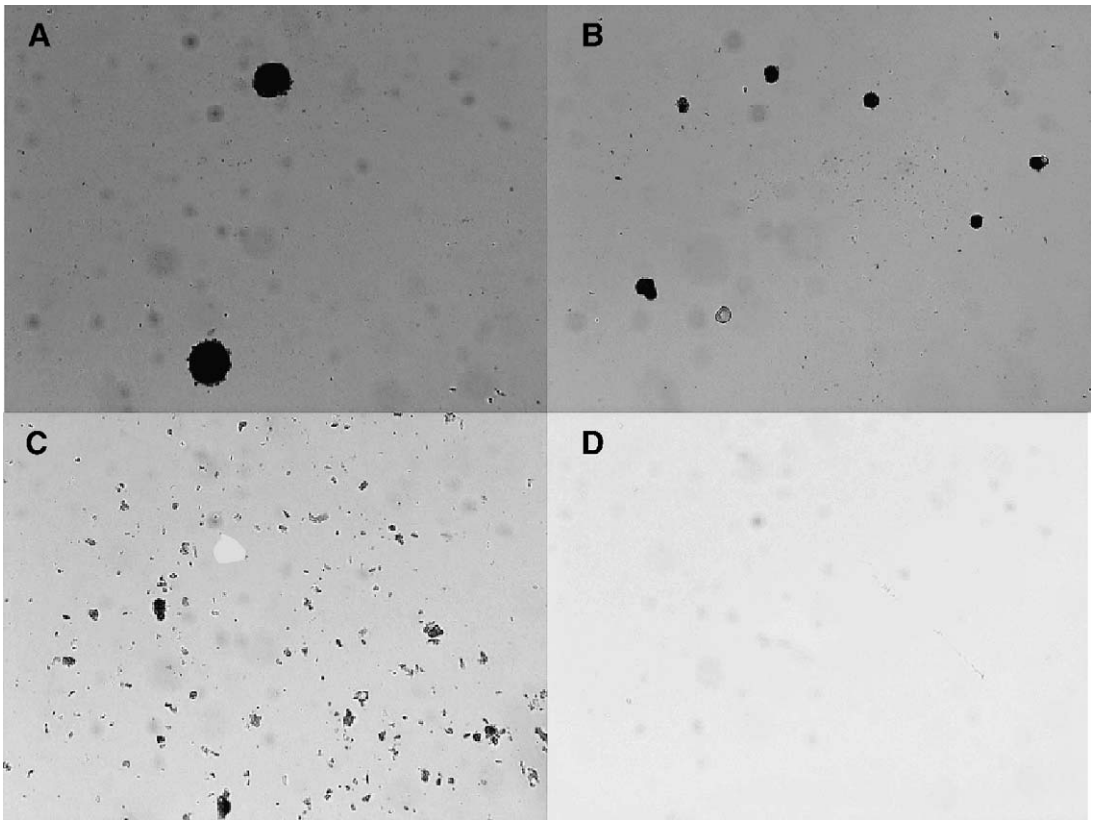


Fig. 5. Optical photomicrographs of intraperitoneal cavity washings after injection of HES microcapsules in female B6D2F1 mice: day 1 (A), day 3 (B), day 5 (C) and day 7 after injection (D); magnification ×40.

Animals were followed for 3 weeks and no inflammatory signs could be observed. The microcapsules seemed subsequently well-tolerated.

Then, the in vivo degradation study was realized: microparticles were injected in i.p. to 21 mice, and each day one group of three mice was sacrificed to evaluate microparticles degradation by light microscopy (Fig. 5). The day after administration, the microparticles stained with Methylene Blue presented a lesser smooth surface (Fig. 5A). Three days later, the microparticles were cracked (Fig. 5B) and at day 5, only some small fragments could be visualized (Fig. 5C). Seven days after the injection, nothing remained observable, which meant that the totality of microcapsules was degraded within a week (Fig. 5D). After i.p. administration, a depot of microcapsules was formed at the injection site which could increase the immune response comparable with the effect seen with other depot adjuvants (Sinha and Thehan, 2003). This interesting result showed that HES microcapsules prepared by interfacial cross-linking could be used to allow a slow release of loaded protein (e.g. antigens); most of the associated protein would only be released after biodegradation of the HES microcapsules.

4. Conclusion

The aim of this work was to study HES microparticles for future in vivo applications. This study demonstrated that these microcapsules showed an important loading capacity for the model protein BSA. CLSM analyses fully demonstrated that the loaded BSA was present at the surface as well as entrapped inside the microparticles. Furthermore, a significant part of BSA (80%) was left to be released after complete degradation of the microparticles. From BSA loading experiments and the in vivo degradation profile of HES microparticles, we could deduce that high amounts of the model protein are expected to be released with a suitable controlled release profile. Moreover, both the absence of cytotoxicity and the observed microparticles tolerance reinforce the suitable in vitro and in vivo characteristics for drug delivery especially to enhance the presentation of antigens. The HES microparticles are currently under further investigations in order to increase immune response against melanoma resistant cancer cells after loading with soluble melanoma proteins.

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