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European Journal of Pharmaceutics and Biopharmaceutics 66 (2007) 11–20
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Cellulose acetate butyrate–pH/thermosensitive polymer microcapsules containing aminated poly(vinyl alcohol) microspheres for oral administration of DNA

Gheorghe Fundueanu [a,b], Marieta Constantin [b], Fabrizio Bortolotti [a], Rita Cortesi [a,*], Paolo Ascenzi [c], Enea Menegatti [a]

[a] Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy
[b] Department of Bioactive and Biocompatible Polymers, "Petru Poni" Institute of Macromolecular Chemistry, Iassy, Romania
[c] Department of Biology and Interdepartmental Laboratory for Electron Microscopy, University "Roma Tre", Rome, Italy

Received 13 April 2006; accepted in revised form 12 September 2006
Available online 22 September 2006

Abstract

The aim of this work is to safely transport bioadhesive microspheres loaded with DNA to intestine and to test their bioadhesive properties. Poly(vinyl alcohol) (PVA) microspheres were prepared by dispersion reticulation with glutaraldehyde and further aminated. These microspheres were firstly loaded with plasmid DNA by electrostatic interactions and then entrapped in cellulose acetate butyrate (CAB) microcapsules for gastric protection. The entrapped PVA microspheres do not have enough force by swelling to produce the rupture of CAB shell, therefore the resistance of microcapsules was weakened by incorporating different amount of the pH/thermosensitive polymer (SP) based on poly(*N*-isopropylacrylamide-co-methyl methacrylate-co-methacrylic acid) (NIPAAm-co-MM-co-MA). This polymer is insoluble in gastric juice at pH 1.2 and 37 °C, but quickly solubilized in intestinal fluids (pH 6.8 and pH 7.4). Therefore, DNA loaded PVA microspheres were not expelled in acidic media but were almost entirely discharged in small intestine or colon. The integrity of DNA after entrapment was tested by agarose gel electrophoresis indicating that no DNA degradation occurs during encapsulation. The percentage of adhered microspheres on the mucus surface of everted intestinal tissue was 65 ± 18% for aminated PVA microspheres without DNA and almost 50 ± 15% for those loaded with DNA. Non-aminated PVA microspheres display the lowest adhesive properties (33 ± 12%). In conclusion DNA loaded microspheres were progressively discharged in intestine. The integrity of DNA was not modified after entrapment and release, as proved by agarose gel electrophoresis. Both loaded and un-loaded aminated microspheres display good bioadhesive properties.
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Keywords: DNA; pH/thermosensitive polymers; Bioadhesive microspheres; Drug delivery systems

1. Introduction

Oral gene delivery is a main goal for numerous biotechnological companies. The main advantages presented by oral gene delivery are the ease of target accessibility, the enhanced patient compliance owing to the non-invasive delivery method, and the possibility of local and systemic gene therapy. To date, most gene delivery strategies have concentrated on the parenteral route of delivery [1–4], and oral administration has been largely ignored with few exceptions [5,6]. The lacking of using oral route for gene

Abbreviations: AIBN, *N*,*N*′-azobisisobutyronitrile; CAB, cellulose acetate butyrate; CDI, *N*,*N*-carbonyldiimidazole; CP, cloud point; CyH, cyclohexane; DC, degree of cross-linking; EC, exchange capacity; GA, glutaraldehide; SP, (pH/thermo)sensitive polymer; LCST, lower critical solution temperature; MA, methacrylic acid; MM, methyl methacrylate; NIPAAm, *N*-isopropylacrylamide; PVA, poly(vinyl alcohol).

* Corresponding author. Department of Pharmaceutical Sciences, University of Ferrara, I-44100 Ferrara, Italy. Tel.: +39 0532291259.
E-mail address: crt@unife.it (R. Cortesi).

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doi:10.1016/j.ejpb.2006.09.002

delivery is mainly due to the large number of hurdles that need to be overcome, such as the acidic pH in the stomach, the presence of nucleases, lipases, and peptidases in the GI tract, and the poor permeability of both genes and gene vectors across the intestinal epithelium.

DNA delivery systems can be classified generally within two main classes: the viral mediated systems (e.g., adeno- and retroviral vectors) [7,8] and the non-viral delivery systems (e.g., neutral or cationic polymers, micro- and nanospheres) [9–11]. Among non-viral transfection agents an attractive class to consider is represented by bioadhesive microspheres. The main advantage of these microspheres is the possibility to design and synthesize microparticles starting from polymers carrying different structural elements that can self-assemble with DNA by electrostatic interactions thus producing vectors with a range of properties [12]. In this view, the most important benefit is the possibility of not exposing nucleic acid molecules to chemical, thermal, and mechanical stresses during microparticle preparation.

In addition, bioadhesive microspheres have been reported to increase the peroral bioavailability of insulin and have been investigated for peroral gene delivery [13]. The increased bioactivity of insulin and of the plasmid DNA can be accounted to the uptake of microspheres by cells lining the small and large intestine epithelium. Bioadhesive microspheres by keeping the drug in the region proximal to its adsorption window allow targeting and localization of the drug at a specific site. However, the major problem of this alternative is to transport the loaded bioadhesive microspheres to small and large intestine avoiding the contact with gastric fluids. The literature presents different strategies for specific drug delivery to the various regions of gastro-intestinal tract and, in particular to the colon [14]. The most part of works are based on microencapsulation with enteric polymers, which are able to release the drug at a particular pH [15,16]. However, in our hands, this method failed because of microsphere swelling during preparation process. We recently reported the encapsulation of cationic-exchange microspheres loaded with tetracycline in cellulose acetate butyrate (CAB) microcapsules [17]. The drug release was possible owing to the higher swelling degree of sulfopropylated dextran resins in intestinal than in gastric fluids, causing the rupture of CAB shell, and the escape of loaded microspheres. The present paper reports a new approach for the preparation of microspheres with cationic surface to improve the intestinal delivery of DNA. In particular, we propose the encapsulation of aminated PVA microspheres loaded with plasmid DNA in CAB microcapsules for intestinal delivery of the nucleic acid. Since aminated PVA resins, contrary to sulfopropylated microspheres, do not swell enough in intestine to produce the rupture of CAB shell, we incorporate in microcapsule composition different amount of pH/thermosensitive polymers (as enteric materials). These polymers weaken the resistance of the CAB shell by their dissolution in the intestinal fluids causing halls in CAB microcapsules and allowing the release of loaded PVA microspheres. In other words, the use of CAB/pH/thermo-responsible shell instead of conventional enteric capsules is more advantageous since the encapsulated PVA microspheres are progressively released through the halls of CAB microcapsules created by dissolution of pH/thermosensitive polymer. Also, the bioadhesive properties of aminated PVA microspheres with and without DNA were tested.

2. Materials and methods

2.1. Materials

PVA ($M_w = 18{,}000$ g/mol; hydrolysis mole = 98.4%) was purchased from Air Products and Chemicals, Inc. (Utrecht, The Netherlands). Cellulose acetate butyrate (CAB), low viscosity, was obtained from Eastman Inc. (Kingsport, Tennesse, USA). Glutaraldehyde (GA) (2.6 M aqueous solution) was supplied by Fluka AG (Seelze, Germany). 3-Dimethylamino-1-propyl amine and *N*, *N*-carbonyldiimidazole (CDI) were purchased from Fluka AG (Buchs, Switzerland). *N*-isopropylacrylamide (NIPAAm), supplied by Sigma-Aldrich Chemical Corp. (St. Louis, MO, USA), was recrystallized with hexane. Methacrylic acid (MA) and methyl methacrylate (MM), supplied by Fluka AG (Buchs, Switzerland), were distilled under reduced pressure before use. *N*,*N*′-azobisisobutyronitrile (AIBN), from Fluka AG (Buchs, Switzerland), was purified in methanol. 1,4-Dioxane, supplied by Fluka AG (Buchs, Switzerland), was purified by refluxing. Chloroform, methylene chloride and cyclohexane were provided from Fluka, AG (Buchs, Switzerland). Plasmid DNA, low molecular weight, from salmon sperm, was supplied by Fluka, AG (Buchs, Switzerland).

2.2. Preparation of PVA microspheres

PVA microspheres were obtained using a cylindrical glass reactor, provided with an anchor type glass stirrer, and a reflux condenser. The reactor was maintained at 50 °C using a thermostatic water bath.

Briefly, 4 g of PVA was dissolved in 20 ml hot water. The solution was acidified with 1.6 ml of 0.5 M H_2SO_4 solution, and then poured in 100 ml of dispersing medium (1,2-dichloroethane) containing 1 g of CAB (as the dispersion agent). This water/organic solvent emulsion was stirred for 30 min (stirring speed = 750 rpm), then 1–2 ml of GA was added, and the cross-linking reaction was carried out for 4 h at 50 °C. The cross-linked microspheres were recovered by filtration through a sintered glass filter, under vacuum. The removal of residuals was performed by washing the microspheres in the following order: 1,2-dichloroethane, acetone, hot water, cold water, and methanol. Then, the microspheres were completely dried by overnight exposure to 60 °C, under vacuum.

2.3. Amination of PVA microspheres

The synthetic scheme for the preparation of aminated PVA microspheres is depicted in Fig. 1. Two grams of PVA microspheres was swollen in 20 ml DMSO containing 1 g CDI and maintained 18 h at 70 °C for activation of hydroxyl groups. Thereafter, 12 ml of 3-dimethylamino-1-propyl amine was added to the suspension and the mixture was left to react for 10 h under stirring at 80 °C. After cooling at room temperature, the microspheres were filtered and consecutively rinsed with DMSO, water, and methanol.

2.4. Determination of microspheres exchange capacity

The exchange capacity of aminated PVA microspheres was determined under dynamic conditions by standard methods [18].

2.5. DNA-binding capacity of aminated microspheres

DNA-free microspheres, prepared using the procedure described above, were loaded as follows. Previously swollen, empty microspheres (stored for 24 h in distilled water) were placed in a chromatographic column and allowed to slowly settle and pack. 100 ml of DNA solution (0.5%, w/v) in phosphate buffer at pH 7.4 (NaH_2PO_4, 20 mM, and Na_2HPO_4, 80 mM) was then passed through the packed column. In order to maximize microsphere loading, an excess of DNA was used with respect to the exchange capacity of the beads. Particularly a 3 mmol DNA to 1 meq exchange capacity ratio was employed. The microspheres were then washed to remove unbound DNA. The amount of associated DNA was calculated evaluating the concentration of free DNA after filtration of the microspheres. DNA determination was performed by UV spectrophotometric analysis (Perkin-Elmer, Norwalk, CT, USA) using a previously constructed calibration curve.

2.6. Synthesis of poly(NIPAAm-co-MA-co-MM)

Synthesis of linear poly(NIPAAm-co-MA-co-MM) was carried out by free radical copolymerization in 1,4-dioxane with AIBN as initiator. Typically, 1.13 g NIPAAm, 0.086 ml MA, 0.2 ml MM, and 0.010 g AIBN were solubilized in 10 ml of 1,4-dioxane. Dried nitrogen was bubbled through the solution for 30 min prior to polymerization. After polymerization at 70 °C for 20 h, the mixtures were precipitated in diethylether, and dried under vacuum. The obtained copolymer was solubilized in distilled water, dialyzed for 5 days at 20 °C (molecular weight cut off 10,000–12,000; Medi Cell International, England), and recovered by lyophilization. This polymer will be further called as "(pH/thermo)sensitive polymer" (SP).

2.7. LCST determination

The temperature dependence of absorbance at 450 nm was measured using a UV–Vis spectrophotometer coupled with a temperature controller. The polymer solutions (1%, w/v) were prepared in water, standard acidic buffer at pH 1.2 (50 mM KCl + 64 mM HCl) and standard isotonic phosphate buffers at pH 6.8 (51 mM NaH_2PO_4 + 49 mM Na_2HPO_4) and pH 7.4 (20 mM NaH_2PO_4 + 80 mM Na_2HPO_4). The heating rate was 2 °C every 10 min and 0.2 °C in the vicinity of the cloud point (CP). The CP was defined as the temperature at the inflexion point in the normalized absorbance versus temperature curve.

2.8. Synthesis of microcapsules containing DNA loaded PVA microspheres

Microcapsules containing loaded PVA microspheres were obtained by an oil/water solvent evaporation method [17] using an open cylindrical reactor ($h = 120$ mm, i.d. = 60 mm), and a three blade turbine impeller.

Typically, 50 mg SP were solubilized in 0.1 ml methanol and the solution was diluted with 1 ml mixture of $CHCl_3$:CH_2Cl_2 (1:1 v/v). Then, 150 mg of CAB was solubilized in the diluted solution and 0.2 ml cyclohexane was added under stirring, as inert solvent. Afterward, 100–200 mg of DNA loaded PVA microspheres was suspended for 5 min in the polymer solution. Finally, the obtained homogeneous suspension was emulsified (stirring speed of 500 rpm) into an external aqueous phase (100 ml, containing 1%, w/v PVA, 88% hydrolyzed, as emulsifier). The encapsulation process started at 25 °C for 5 s, then the temperature was raised up to 40 °C, and the process continued for further 30 min. The obtained microcapsules were separated by filtration, washed with 100 ml warm water (40 °C),

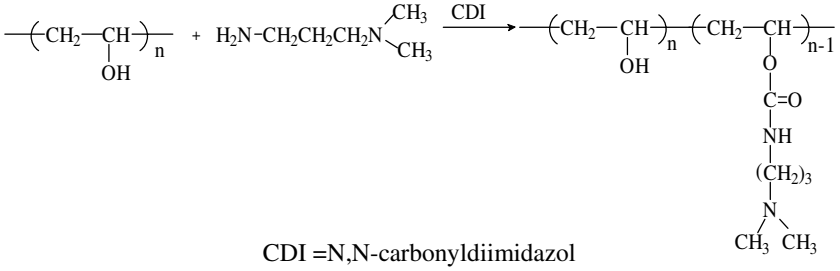

Fig. 1. Synthetic scheme for the preparation of aminated PVA microspheres.

and finally dried under vacuum at 40 °C. During microcapsule preparation, at different intervals of time, microphotographs were taken using an optical microscope equipped with a digital camera.

2.9. Determination of DNA loading

DNA loading of the microcaspules was determined after dissolution of 25 mg of microcapsules in 2 ml chloroform, followed by extraction with 100 ml phosphate buffer, pH 7.4, containing 1 M NaCl (at this ionic strength the dislocation of DNA is total). The amount of DNA was determined by UV–Vis spectrophotometric analysis using samples previously centrifuged for 10 min at 10,000 rpm, and expressed as weight of DNA (mg)/weight of microcapsules (mg) × 100. The encapsulation efficiency of the DNA was calculated as the ratio between the actual DNA content and the theoretical DNA content, and expressed as percentage.

2.10. Morphological and dimensional analysis

Microcapsule morphology was evaluated by optical and electron microscopy. Dried microcapsules were analyzed at 15–20 kV by scanning electron microscopy (SEM) (Cambridge S 360) after metallization by gold coating (Edwards Sputter coating S 150). Size and size distribution were evaluated by optical microscopy using an inverted microscope (Nikon Diaphot, Tokyo, Japan) equipped with a digital camera. Microcapsule size was determined by examining the microcapsule diameter on digital photomicrographs, considering at least 200 microspheres for each sample.

2.11. Swelling degree

The increase in volume of the PVA microspheres (free and loaded with DNA) was determined at equilibrium, placing microspheres in an acid buffered solution at pH 1.2 (50 mM KCl + 64 mM HCl), in an isotonic phosphate buffered solution at pH 7.4 (20 mM NaH_2PO_4 + 80 mM Na_2HPO_4) or in demineralized water. The volume of the swollen microspheres (V_s) reported to the dried volume (V_d) measured by placing the microspheres in a graduated cylinder ($d = 12$ mm) was defined as the swelling factor ($q = V_s/V_d$).

2.12. In vitro DNA release studies

In vitro DNA release studies were determined by the bath method [19], using different buffered solutions simulating the gastric juice (pH 1.2, KCl + HCl) or the intestinal fluid (isotonic buffered solutions at pH 6.8 and pH 7.4, $NaH_2PO_4 + Na_2HPO_4$).

The samples were dispersed in flasks containing buffered solutions at 37 ± 0.5 °C, under gentle stirring (50 rpm). Samples of the receiving buffer were withdrawn at different time intervals and the drug content was determined by spectrophotometric analysis. The same volume of the fresh receiving buffer was added to replace the volume of the withdrawn samples. During the release process, the morphological changes in the structure of CAB microcapsules were recorded by an optical microscope equipped with a digital camera.

2.13. Stability of DNA

The stability of DNA after encapsulation and release from microspheres was investigated by agarose gel electrophoresis. DNA electrophoresis was performed in 3% (w/v) agarose gels containing 0.5 μm ethidium bromide for visualization, for 3 h at 25 mV constant current. The relative band migration was determined, after gel staining with ethidium bromide.

2.14. Bioadhesion tests

Bioadhesive properties of unencapsulated PVA microspheres and aminated PVA microspheres with and without DNA were evaluated using the everted sac technique [20] with minor modifications for an easier handling of the small intestine during the filling operation as we previously reported [21]. Unfasted rats (white adult males, similarly nourished, which were grown in normal laboratory conditions) were killed and intestinal tissue was excised and flushed with 10 ml ice-cold isotonic phosphate buffer (PB), pH 7.2, containing 2 mg/ml glucose. Six centimeter segments of jejunum were everted using a glass tube with a conical end, and lightly washed with PB to remove the contents. One ligature was placed at the end of the segment located near to the conical end of the tube. Through the opposite end of the tube 1.0–1.5 ml PB was poured until the sac was filled, thereafter the segment end was tightly tied. The intestinal tissue was maintained at 4 °C prior to incubation. The sacs were introduced into a 15-ml glass tube containing 60 mg of microspheres and 5 ml PB, incubated at 37 °C and agitated end-over-end. After 30 min, the sacs were removed, then the not attached microspheres were recovered by filtration and dried. The percent of the attached microspheres was calculated by the difference between the initial amount of microspheres and the amount of not attached microspheres. The experiment was performed in duplicate.

3. Results and discussions

3.1. Preparation and characterization of aminated PVA microspheres

PVA is a biocompatible and non-toxic polymer that is frequently used in biomedical applications, such as implants [22], soft contact lenses [23], artificial organs [24], and protein immobilization [25]. In addition, PVA is able to form hydrogels branded by high degree of swelling in reason of its hydrophilic character. Therefore, PVA is

one of the synthetic polymers used in bioadhesive formulations [26] improving the mucosal adsorption of DNA.

Moreover, the presence of hydroxyl groups capable of cationization makes PVA microspheres suitable for self-assembly with DNA by electrostatic interactions.

PVA microspheres were produced by suspension crosslinking procedure with glutaraldehyde. The reaction was carried out in a water/organic solvent suspension using a 18.5% (w/v) acidified aqueous polymer solution. The produced microspheres were characterized by electron microscopy showing good spherical geometry, smooth surface and a range dimension comprised between 50 and 160 μm (data not shown).

After preparation, crosslinked PVA microspheres were aminated and then loaded with DNA. It must be noticed that the high percentage of loaded DNA was obtained by passing DNA over the swollen microspheres under dynamic conditions (Table 1). In addition, due to the low charge density (0.7 meq/g) these microspheres provided good access of low molecular weight DNA fragments. It must be noticed the difference of swelling degree between free and loaded microspheres in aqueous phase and at different pH (Table 1), representing one of the key factors for the successful encapsulation within the microcapsules, and for the modulation of the DNA release profiles.

3.2. Preparation of microcapsules

The main disadvantage that need to be overcome for oral gene delivery is the acidic pH in the stomach that leads to depurination of DNA. In this view, DNA loaded PVA microspheres were included in CAB microcapsules following an oil/water solvent evaporation method previously reported [17].

However, the entrapped microspheres do not have enough force to produce, by swelling, the rupture of CAB shell in intestinal fluid (see release studies), therefore the resistance of the CAB shell was weakened by incorporating different amounts of the pH/thermosensitive copolymer, that is insoluble in the gastric juice at pH 1.2, $T = 36$ °C, but quickly solubilizes in intestinal fluids (pH 6.8 and 7.4) at 36 °C (Fig. 2).

A first set of experiments was performed to determine the optimal standard parameters for microcapsule preparation such as type of solvents and polymer concentration,

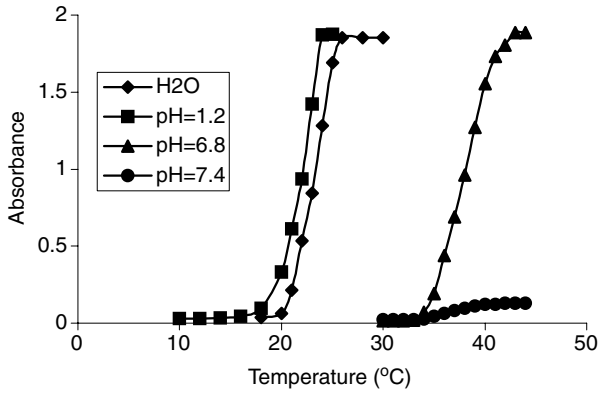

Fig. 2. LCST profiles of poly(NIPAAm-co-MM-co-AM) in water and different buffers (concentration of polymer solution was 1%, w/v). The values are means of three independent measurements that deviated 1–3%.

CAB/SP ratio, stirring speed, solvent evaporation temperature and the amount of entrapped PVA microspheres. The best results in terms of recovery, shape, and encapsulation efficiency were obtained using $CHCl_3$:CH_2Cl_2 (1:1, v/v), as volatile solvents, a stirring speed of 500 rpm, a solvent evaporation temperature of 40 °C, a polymer concentration (CAB/SP) of 20% (w/v) and a CAB/SP ratio of 3/1 (w/w). The addition of 10% (v/v) methanol was strictly necessary to assure a complete solubilization of SP in the solvent mixture. In these conditions microcapsules with properties suitable to pharmaceutical applications (i.e., spherical shape, percentage of recovery, encapsulation efficiency and drug release) were obtained.

In a second attempt, the possibility to increase the percentage of DNA encapsulation was investigated (see Table 2). Initially, 100 mg of DNA loaded PVA microspheres was encapsulated with high percentage of recovery within CAB/SP microcapsules (sample M #1) and was characterized by an almost spherical shape. By the contrary, the microcapsules obtained with a payload of 150 mg of PVA (sample M #2) showed an irregular geometry, a percentage of recovery minor of 45% and appeared often broken probably in reason of the swelling of PVA microspheres located at the peripheral zone of CAB/SP microcapsules before film formation. In order to overcome these drawbacks, an increase of the volume of the polymer solution (without to decrease its viscosity, that is one of the key factors for the successful encapsulation of microspheres) was required.

Table 1
Main characteristics of aminated PVA microspheres in the absence and presence of DNA

Sample	Diameter (μm)	DC (PVA/GA molar ratio)[a]	EC (meq/g)[b]	DNA content (%, w/w)	Efficiency (% from theoretical)	Swelling degree $q = V_s/V_d$		
						H_2O	pH 1.2	pH 7.4
I_1	50–160	35/1	0.74 ± 0.04			4.28 ± 0.21	5.02 ± 0.20	3.85 ± 0.23
I_1 + DNA				14.75 ± 0.79	76.5 ± 4.1	2.24 ± 0.16		
I_2	50–160	17.5/1	0.72 ± 0.05			3.16 ± 0.22	3.98 ± 0.18	2.88 ± 0.14
I_2 + DNA				12.44 ± 0.85	65.8 ± 4.5	2.08 ± 0.15		

Data are results of three independent experiments ± SD.
[a] DC, degree of crosslinking expressed as PVA/GA molar ratio.
[b] EC, exchange capacity.

Table 2
Preformulatory studies: experimental parameters and microcapsule characteristics[a]

Code	CyH (ml)[b]	CH_3OH (ml)	Amount of CAB (mg)	Amount of SP (mg)[c]	Amount of I_1 + DNA (mg)	Recovery (%)	DNA content in microcaps. (%, w/w)	Encaps. efficiency (%)	Diameter (μm)	Note
M #1	0	0.1	150	50	100	90.2 ± 7	5.1 ± 0.2	103.8 ± 4.1	220 ± 15	Almost spherical shape
M #2	0	0.1	150	50	150	44.5 ± 6	–	–	245 ± 20	Irregular shape, some broken particles
M #3	0.2	0.1	150	50	150	88.0 ± 6	6.45 ± 0.15	102.0 ± 2.4	245 ± 18	Spherical shape, no aggregation
M #4	0.2	0.1	150	50	200 (I_2 + DNA)	80.2 ± 5	6.41 ± 0.30	103.0 ± 4.8	268 ± 21	Spherical shape, no aggregation

Data are results of three independent experiments ± SD.
[a] Solvent: $CHCl_3/CH_2Cl_2$ = 0.5/0.5 ml; stirring speed = 500 rpm; T = 40 °C.
[b] CyH = cyclohexane.
[c] SP = (pH/thermo)sensitive polymer.

Therefore, different percentages of cyclohexane ranging from 10 to 40% were added. As we anticipated, cyclohexane is miscible with $CHCl_3$ and CH_2Cl_2, but is non-solvent for CAB and SP. The addition of 20% (v/v) of the inert solvent gives several advantages.

(i) The increase of the volume of the organic phase with no significant decrease of the viscosity [17], resulting in an increase of the entrapment up to 200 mg loaded PVA microspheres (sample M #4 in Table 2, and samples M #5–M #8 in Table 3). (ii) The permanence of the inert solvent in the pores of the hardened microcapsules until and after complete evaporation of the volatile solvents (CAB microcapsules displaying floating properties) due to the higher boiling point of cyclohexane with respect to $CHCl_3$ and CH_2Cl_2. In these conditions, the intimate contact between encapsulated microspheres and aqueous dispersion phase is obstructed (Fig. 3A), and the obtained microcapsules are spherical (Fig. 3B). (iii) The removal of the inert solvent under vacuum at 40 °C generates a uniform porosity in the matrix of CAB/SP microcapsules (Fig. 3C).

The encapsulation of PVA microspheres took place in the dried state as proven by the absence of any free space between encapsulated microspheres and CAB/SP network as shown in Fig. 3C. Finally, the influence of cross-linking degree and CAB/SP ratio on microcapsule preparation and characteristics were investigated. Higher degree of cross-linking led to a lower swelling degree and therefore to an easier entrapment (higher microsphere recovery) (sample M #4 in Table 2). However, the amount of electrostatic linked DNA is lower due to the less accessible site of interaction in a more contracted matrix (Table 1). A higher proportion of SP in CAB microcapsules led to a decrease of the recovery as well as of the DNA content in microcapsules (Table 3). Moreover, it was not possible to obtain microcapsules from sole SP or CAB/SP mixture (1:1, w/w) even if the solvent evaporation temperature was above the LCST. In fact, the presence of a high SP amount decreases the viscosity of the solution, and SP even above LCST is less hydrophobic than CAB.

3.3. In vitro release studies

The encapsulation of DNA loaded PVA microspheres was mainly due to a combination between CAB and SP having excellent film forming properties and being insoluble in slightly acidic medium of dispersion at the

Table 3
Influence of the CAB/IP ratio on microcapsule characteristics[a]

Code	CyH (ml)[b]	CH_3OH (ml)	Amount of CAB (mg)	Amount of SP (mg)[c]	Amount of I_1 + DNA (mg)	Recovery (%)	DNA content in microcaps. (%, w/w)	Encaps. efficiency (%)	Diameter (μm)	Note
M #5	0.2	0.1	200	0	200	85.5 ± 5	8.1 ± 0.31	109.9 ± 4.2	340 ± 28	Spherical shape, no aggregation
M #6	0.2	0.1	175	25	200	77.3 ± 7	7.9 ± 0.22	107.2 ± 3	275 ± 19	Spherical shape, no aggregation
M #7	0.2	0.1	150	50	200	72.1 ± 6	7.4 ± 0.15	100.4 ± 2	245 ± 14	Spherical shape, no aggregation
M #8	0.2	0.1	125	75	200	60.1 ± 4	5.9 ± 0.18	80 ± 2.4	228 ± 23	Almost spherical shape, pelicles and broken microcapsules

Data are results of three independent experiments ± SD.
[a] Solvent: $CHCl_3/CH_2Cl_2$ = 0.5/0.5 ml; stirring speed = 500 rpm; T = 40 °C.
[b] CyH = cyclohexane.
[c] SP = (pH/thermo)sensitive polymer.

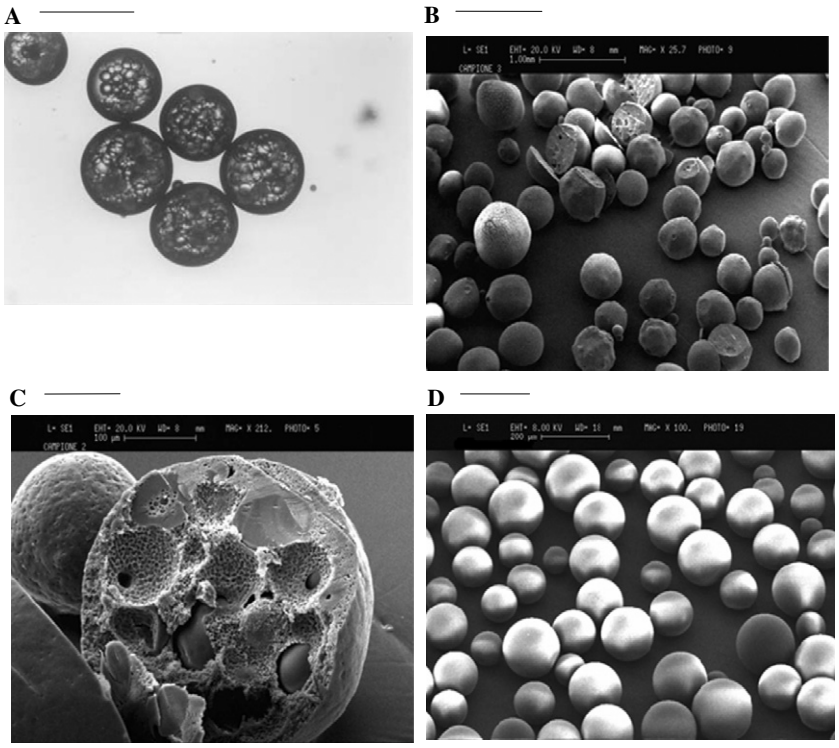

Fig. 3. Optical photomicrograph taken during microcapsule formation (sample M #7). Photomicrograph was taken after 15 min (A). Scanning electron micrographs of CAB/SP microcapsules containing loaded PVA microspheres (sample M #7): general view (B), cross-section (C). For comparison scanning electron micrographs of loaded PVA microspheres (D) are depicted. The bars correspond to 300, 1000, 100 and 200 μm in panel A, B, C and D, respectively.

preparation temperature. The presence of SP induces the formation of holes within the CAB shell allowing the escape of loaded PVA microspheres (Fig. 4). In addition, the absence of an intimate contact between swellable entrapped microspheres and aqueous dispersion medium was obtained using an appropriate polymer concentration

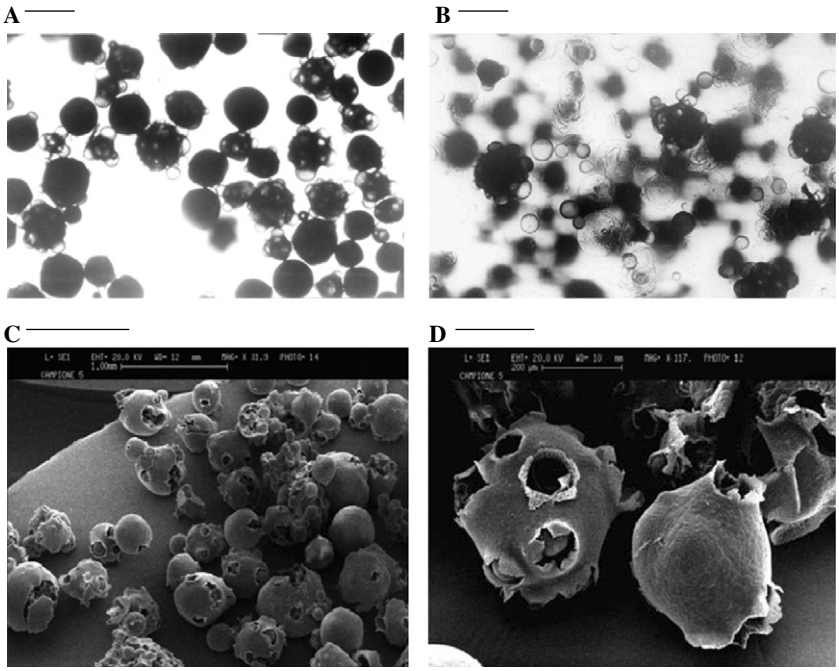

Fig. 4. Optical (A, B), and scanning electron photomicrographs (C, D) taken during release study (sample M #7). Photomicrographs were taken at 1 h (A, C), and 4 h (B, D) after incubation in phosphate buffer at pH 7.4, $T = 37$ °C. The smaller and transparent particles represent released PVA microspheres. The bars correspond to 250 μm in panel A and B, and to 1000 and 200 μm in panel C and D, respectively.

and the presence of an inert solvent until the reaching of harded microcapsules. On the opposite, the DNA release should be favoured by the access of the release fluids within the encapsulated PVA microspheres occurring through the microcapsule pores after cyclohexane removal. In particular, DNA is promptly released from unencapsulated PVA microspheres (Fig. 5A, Table 4) while it is faster released in phosphate buffer. More precisely, DNA release in phosphate buffer at pH 7.4 and 36 °C, where SP is highly soluble, is faster with respect to the release at pH 6.8, where SP is less soluble. As demonstrated by data reported in Fig. 5B and Table 4, at acidic pH, DNA is very slowly and poorly released. Particularly, only the microspheres localized at the periphery of microcapsules can be subjected to DNA release. The increase of the pH from 1.2 to 7.4 induces a dramatic enhancement of the DNA release owing to the dissolution of IP from the microcapsule shell.

The absence of SP in the shell of microcapsules slowers the release of DNA. Then, the presence of SP in the CAB microcapsules network induces the faster disintegration of microcapsules and an easier escape of the loaded microspheres (Fig. 5C, Table 4). After dissolution of SP the escape of microspheres from the CAB matrix takes place by their own swelling. When the microspheres come in contact with the phosphate buffer, the pressure created by their swelling breaks the CAB film and they start to be released. High degree of crosslinking signifies low swelling degree, on the contrary, lower crosslinking degree means higher degree of swelling. Accordingly, the release rate of microspheres and therefore of DNA will be faster for the microspheres with a lower crosslinking degree (Fig. 5D, Table 4). Data given in Fig. 5, panels A, C, and D fit very well with the two-exponential kinetics (Eq. (1) in Table 4; $R \simeq 0.999$) while is very poor the data analysis according to one-exponential kinetics (Eq. (2) in Table 4; $R \simeq 0.960$). By contrast, data given in panel B fit very well to one-exponential kinetics (Eq. (2) in Table 4; $R \simeq 0.999$) and data analysis according to two-exponential decay is very poor (Eq. (1) in Table 4; $R \simeq 0.960$). These data suggest that DNA release follows

Table 4
Values of kinetic parameters obtained from the best fit of data given in Fig. 5 according to Eq. (1) or (2)

Panels	Symbols	k_1 (h^{-1})	Y_1 (%)	k_2 (h^{-1})	Y_2 (%)	k (h^{-1})	Y (%)	Eq.
A	■	4.3	83	0.27	17	–	–	1
	×	2.2	24	0.13	76	–	–	1
	▲	1.1	35	0.049	65	–	–	1
B	○	–	–	–	–	0.17	96	2
	●	–	–	–	–	0.85	15	2
C	▲	2.2	23	0.13	77	–	–	1
	●	0.90	26	0.048	74	–	–	1
	◆	0.58	18	0.0048	82	–	–	1
D	▲	2.2	23	0.13	77	–	–	1
	◆	0.53	41	0.045	59	–	–	1

Eq. (1) $Y_{app} = Y_1 \times (1-e^{-k1t}) + Y_2 \times (1-e^{-k2t})$; Eq. (2) $Y_{app} = Y \times (1-e^{-kt})$.

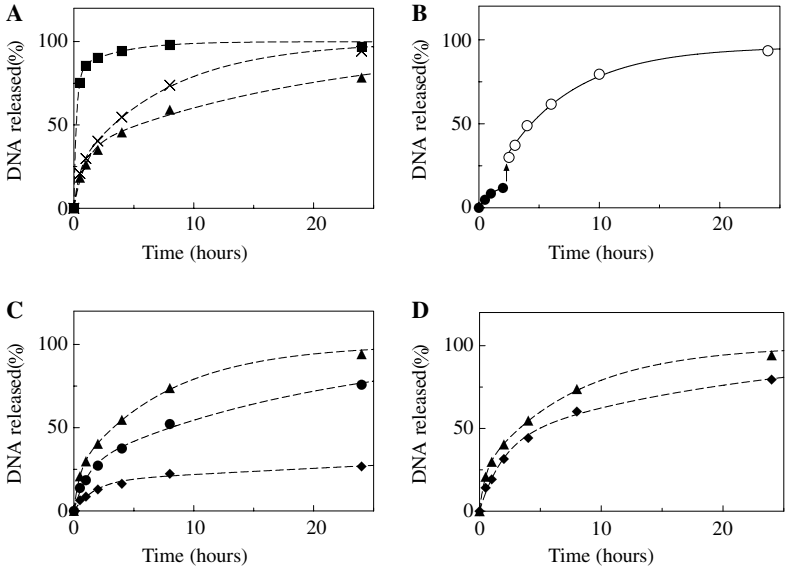

Fig. 5. Panel A. Release profiles of DNA from: unencapsulated PVA microspheres, in isotonic phosphate buffer at pH 7.4 (sample I_1 + DNA) (■); encapsulated PVA microspheres in CAB microcapsules with 25% (w/w) SP (sample M #7), in isotonic phosphate buffer at pH 6.8 (▲), and pH 7.4 (×). The dashed lines were calculated according to Eq. (1) with values of parameters given in Table 4. Panel B. DNA release profile from encapsulated PVA microspheres (sample M #7) after incubation in acidic buffer at pH 1.2 for 2 h (●), and then transferred in isotonic phosphate buffer, pH 7.4 (○). The continuous lines were calculated according to Eq. (2) with values of parameters given in Table 4. The arrow indicates the pH change from pH 1.2 to pH 7.4. Panel C. Release profiles of DNA from: encapsulated PVA microspheres in CAB microcapsules without SP (sample M #5) (◆), encapsulated PVA microspheres in CAB microcapsules with 12.5% (w/w) SP (sample M #6) (●), and with 25% (w/w) SP (sample M #7) (▲). All data were obtained at pH 7.4 The dashed lines were calculated according to Eq. (1) with values of parameters given in Table 4. Panel D. Release profiles of DNA from microcapsules containing loaded PVA microspheres with different crosslinking degree: 2.7% (w/w) (sample M #7) (▲), and 5.5% (w/w) (sample M #4) (◆). All data were obtained at pH 7.4. The dashed lines were calculated according to Eq. (1) with values of parameters given in Table 4.

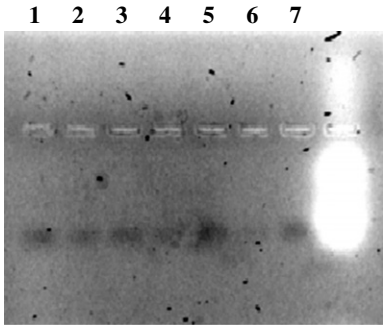

Fig. 6. Agarose gel electrophoresis of DNA-containing fractions collected during release experiments from CAB microcapsules. The release experiments were carried out in isotonic phosphate buffer, pH 7.4. The samples were withdrawn at different time intervals: lanes 1–6: DNA in fractions collected at 30 min, and 1, 2, 4, 8, and 24 h. Lane 7: DNA control sample (1 μg/ml in isotonic phosphate buffer).

a biphasic process although, both erosive and diffusive mechanisms may be involved to a different extent depending on the experimental conditions.

3.4. DNA stability

The stability of DNA after association and encapsulation was evaluated by agarose gel electrophoresis of the released DNA. The relative migration band was determined after staining the gels with ethidium bromide. Fig. 6 shows agarose gel electrophoresis of DNA-containing fractions collected after 30 min and 1, 2, 4, 8 and 24 h of permanence in isotonic phosphate buffer at pH 7.4. In all cases the migration of DNA resulted in a single band similar to the control sample indicating that no DNA degradation occurs during encapsulation and release.

3.5. In vitro evaluation of bioadhesion

The percentage of adhered microspheres on the mucus surface of everted intestinal tissue was $65 \pm 18\%$ for aminated PVA microspheres without DNA and almost $50 \pm 15\%$ for those loaded with DNA. Non-aminated PVA microspheres display the lowest adhesive properties ($33 \pm 12\%$). Therefore, on the basis of these values we could suggest that specific interactions between aminated PVA microspheres and sialic acids located in the mucus layer play a significant role in bioadhesion [27]. Moreover, aminated PVA microspheres devoid of DNA display the highest swelling degree allowing a better intimate contact with the mucus layer. It must be noticed that even aminated microspheres loaded with DNA still have around half of amino groups free, able to interact with sialic acid side chains. Besides, during the adhesion test a fraction of DNA should be dislocated to the phosphate buffer. However, the percentage of adhered microspheres is apparently not so high compared with the total amount of the microspheres used for the experiment. The small percentage could be due to the short time of incubation required by the standard bioadhesion test (30 min), that probably is not sufficient to assure a complete interaction between the two opposite charges. On the other hand, loaded PVA microspheres display a diminished value of the swelling degree and therefore a reduced accessibility of the free amino groups. In the case of PVA microspheres, hydrogen bonding between hydroxyl groups of PVA and oligosaccharide side chains from mucus layer probably assures the bioadhesion of PVA microspheres.

4. Conclusions

Aminated PVA microspheres loaded with plasmid DNA by electrostatic interactions were successfully encapsulated in CAB/SP microcapsules by the oil/water solvent evaporation method. The entrapped PVA microspheres do not have enough force by swelling to produce the rupture of the CAB shell, therefore the resistance of microcapsules was weakened by incorporating different amounts of SP. This copolymer is insoluble in the gastric juice at pH 1.2 and 36 °C, but quickly solubilized in the intestinal fluids (i.e., at pH 6.8 and pH 7.4). Therefore, DNA loaded PVA microspheres are not expelled in acidic media but are almost entirely discharged in small intestine or colonic tract. The integrity of DNA after entrapment was tested by agarose gel electrophoresis indicating that no DNA degradation occurs during encapsulation and release.

Acknowledgements

This study was partly supported by grants from the Ministry for Foreign Affairs of Italy and the Ministry for Education and Research of Romania.

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