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Preparation and characterization of cationic microspheres for gene delivery

E. Esposito [a], S. Sebben [a], R. Cortesi [a], E. Menegatti [a], C. Nastruzzi [b,*]

[a] *Dipartimento di Scienze Farmaceutiche, Università di Ferrara, Ferrara, Italy*
[b] *Istituto di Chimica e Tecnologia del Farmaco, Università di Perugia, via del Liceo, 06100 Perugia, Italy*

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Abstract

The production and characterization of cationic microparticles based on Eudragit RS and cationic agents (i.e. a cationic acrylic polymer and three different cationic surfactants) for the delivery of nucleic acids is here described. It was found that morphological and dimensional characteristics of microparticles were influenced by the type and concentration of cationic agent employed and by some experimental parameters such as stirring speed, emulsifying agent and type of rotor. The desoxiribonucleotide Defibrotide (DFT) was associated with positively charged microparticles and its in vitro release kinetics from microparticles were determined. A study of the in vitro toxicity of cationic microparticles on cultured human cell line K562 was also performed, demonstrating that $DDAB_{18}$ microparticles display very low cytotoxicity.

Keywords: Cationic microparticles; Methacrylic copolymer; Controlled release; Gene therapy; Defibrotide (DFT)

1. Introduction

Recently the use of antisense and gene transfection protocols has assumed a very important role as experimental therapeutic approach for genetic, neoplastic and infectious diseases (Uhlmann and Peyman, 1990; Behr, 1993; Tomlinson and Rolland, 1996). Gene transfection, based on the introduction of normal exogenous genes into target cells, enables the replacement of the defect gene product thus restoring normal cell function (Felgner et al., 1987; Hickman et al., 1994). In addition, antisense oligonucleotides can be used to inhibit both in vitro and in vivo protein synthesis (Mirabelli et al., 1991; Neckers et al., 1992; To and Neiman, 1992). More generally, different nucleic acid molecules have been considered to modulate gene transcription providing opportunities to either replace the missing/defect gene or arrest the expression of specific genes. However, after administration nucleic acid molecules should remain stable in order to reach the target cells and to exert a pharmacological effect; in this respect both antisense and transfection technologies require reliable and efficient systems for their delivery into target cells. Different non-viral

* Corresponding author. Tel.: +39-75-5855158; fax: +39-75-5847469; www.unipg.it.
E-mail address: nas@unipg.it (C. Nastruzzi)

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approaches have been proposed, such as neutral or cationic liposomes and polymeric microparticles (Gregoriadis, 1988; Janoff, 1992; Thierry et al., 1992; Cortesi et al., 1994).

In particular microparticles could offer a number of advantages with respect to other delivery systems since: (a) they maintain unaltered their chemicophysical characteristics for long periods allowing long-term storage; (b) depending on their composition they can be administered through different ways (oral, intramuscular or subcutaneous); and (c) they are suitable for industrial production.

Microparticles can in principle vehiculate nucleic acids in two ways: (a) DNA can be physically entrapped in the polymeric matrix of the particle; or (b) DNA can be bound through electrostatic interactions to the positive charged surface of cationic particles, as shown in the schematic representation reported in Fig. 1.

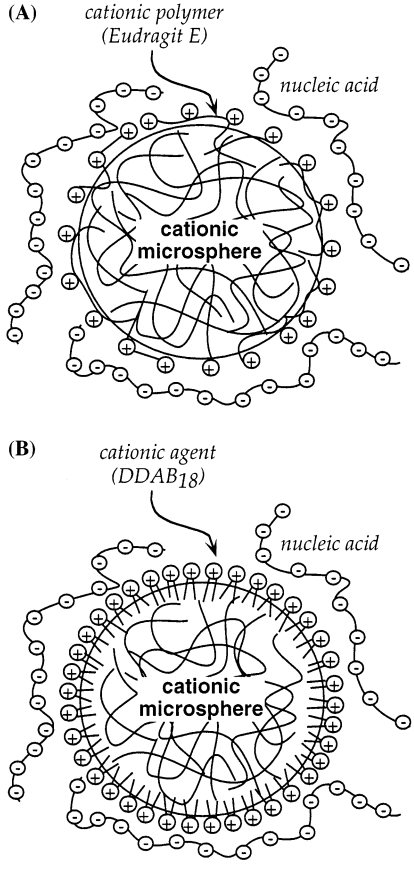

Fig. 1. Schematic representation of the hypothetical association of single or double stranded nucleic acids to positively charged microparticles. (A) Microsphere constituted of Eudragit RS and Eudragit E; (B) microsphere constituted of Eudragit RS and $DDAB_{18}$.

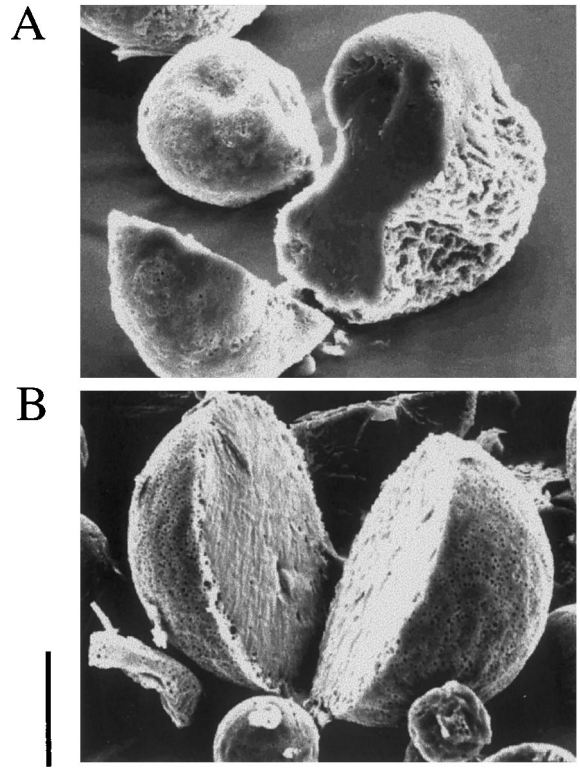

Fig. 2. Scanning electron micrographs of external and internal morphology of Eudragit RS microparticles containing 35% (A) or 30% (B) of Eudragit E. Bar corresponds to 52 and 36 μm, respectively.

The association of DNA molecules to preformed cationic microparticles could provide two important benefits. Firstly, DNA is not exposed to the chemical, thermal or mechanical stresses often present in the production of microparticles, and secondly the association of DNA to microparticles can be performed extemporarily immediately before the administration (Felgner, 1990; Hug and Sleight, 1991). In this way, both microsphere and DNA can be maintained separated in a sterile lyophilized form, avoiding possible long-term stability problems. In order to

confer a positive charge to the microparticle surface different approaches are proposed in this paper using: (a) a polymeric mixture constituted of an uncharged copolymer and a cationic one, or (b) a not entirely polymeric mixture constituted of a neutral copolymer plus a cationic surfactant.

As neutral polymer Eudragit® RS 100 was chosen, a methacrylate copolymer constituted of acrylic and methacrylic acid esters. Eudragit® RS 100 is able to form slightly permeable films, having a low content of quaternary ammonium groups (Lehmann et al., 1989), and it can be used for the production of sustained release formulations (Otsuka et al., 1993; Kim et al., 1994). With regard to charged components, in this study the cationic methacrylic copolymer Eudragit E 100 or different cationic surfactants, namely cetyltrimethyl-ammonium bromide (CTAB), didodecyl-dimethyl-ammonium bromide ($DDAB_{12}$), and dioctadecyl-dimethyl-ammonium bromide ($DDAB_{18}$), were alternatively employed.

Summarizing, this paper describes (a) the production of cationic microparticles by an oil in water solvent evaporation method (Esposito et al., 1997), (b) the characterization of mean dimensions and morphology of the obtained microparticles together with their recovery efficiency, (c) the determination of percentage of association of the desoxiribonucleotide Defibrotide (DFT) and its release kinetics from microparticles, and finally (d) the in vitro assay of microparticles' cytotoxicity on erythroleukemic K562 cells.

Table 1
Effect of Eudragit E concentration on the production of microparticles composed of Eudragit RS and Eudragit E[a]

Eudragit E, % (w/w)[b]	Positive charge (μM)	Mean diameter (μm)	External morphology	Internal morphology	Recovery[c]
0	0	30	Spherical shape, microporous surface	Compact	88
7.5	2.4	33	Spherical shape, microporous surface	Compact	85
15	4.9	44	Spherical shape, microporous surface	Compact	78
22.5	7.3	45	Spherical shape, microporous surface	Compact	86
30	9.7	47	Spherical shape, microporous surface	Compact	82
35	11.3	49	Elissoidal shape, microporous surface	Compact	81
40	12.9	–	Non-spherical shape, large clumps	Collapsed structure	82

[a] Emulsifying agent concentration was 1%, w/w. Stirring speed was always 250 rpm.
[b] Percentage (% w/w) of Eudragit was calculated with respect to total amount of polymer (mg) solubilized in the external phase.
[c] Percentage (% w/w) of isolated microparticles with respect to the initial amount of polymer used for microparticles production.

Table 2
Effect of stirring speed on the production of microparticles composed of Eudragit RS and Eudragit E[a]

Stirring speed (rpm)	Mean diameter (μm)	External morphology	Internal morphology	Recovery[b]
250	47	Spherical shape, microporous surface	Compact	82
500	31	Ellipsoidal shape, microporous surface	Compact	80
750	24	Ellipsoidal shape, porous surface	Compact	76
1000	21	Ellipsoidal shape, microporous surface	Compact	64

[a] Eudragit E concentration was always 30%, w/w. Emulsifying agent concentration was 1%, w/w.
[b] Percentage (% w/w) of isolated microparticles with respect to the initial amount of polymer used for microparticles production.

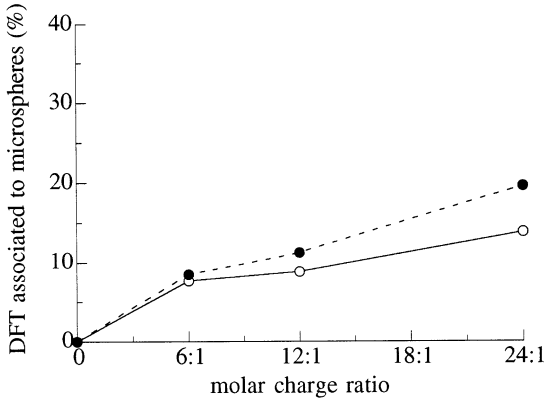

Fig. 3. Percentages of DFT association to cationic microspheres containing 22.5% (○) or 30% (●) Eudragit E.

2. Materials and methods

2.1. Materials

The polymers used for microparticle preparation were the acrylic resins Eudragit RS 100 and Eudragit E 100 from Rohm Pharma (Darmstadt, Germany). The cationic surfactants dioctadecyl-dimethyl-ammonium bromide ($DDAB_{18}$), cethyl-trimethyl-ammonium bromide (CTAB) and didodecyl-dimethyl-ammonium bromide ($DDAB_{12}$) were from Fluka (Buchs, Switzerland). The single stranded polydesoxyribonucleotide DFT was from Crinos, Como, Italy.

2.2. Production of cationic microparticles

Microparticles were produced by the 'solvent evaporation method'. Briefly 500 mg of a polymer and cationic agent mixture were dissolved in 5 ml of CH_2Cl_2. The mixture was emulsified with 100 ml of an aqueous phase containing 1% (w/v) of 88% hydrolysed polyvinyl alcohol (PVA) (Airvol 205, Air Products, PA, USA) as dispersing agent. The obtained emulsion was maintained under continuous stirring with a three-blade turbine impeller Eurostar Digital (Ika Labortechnick, Germany) at 250, 500, 750 or 1000 rpm. At different time intervals, samples were observed microscopically up to complete evaporation of CH_2Cl_2, usually occurring in 3–5 h. Microparticles were then isolated by filtration.

2.3. Optical and electronic microscopy analysis

Microparticle morphology, size and size distributions were determined by optical and electron microscopy observations. For the optical analysis an optical microscope Diaphot (Nikon, Japan) was employed.

For the electronic analysis, microparticles were metallized by gold coating (Edwards Sputter coating S150) and analysed at 15–20 kV by a scanning electron microscope (SEM) 360 Stereoscan (Cambridge Instruments, Cambridge, UK).

2.4. Association of DFT to microparticles

Cationic microparticles were suspended in 3 ml of borate buffer, pH 7.4 containing 250 μg/ml of DFT. The suspension was then maintained under mixing for 12 h using an orbital Ika stirring motor (Labortechnik K Mot, Germany), at 125 rpm. The amount of associated DFT was calculated by evaluating the concentration of the free DFT in the borate buffer after separation of the microspheres. The determination was performed by diluting the borate buffer 1:10 v/v and evaluating DFT concentration by UV spectrophotometric analysis (Perkin Elmer, Norwalk, USA).

2.5. Release kinetics of DFT from cationic microspheres

In vitro release kinetics of DFT from cationic microspheres was determined by a 'dynamic flow' method (Nastruzzi et al., 1993). Briefly 50 mg of cationic microspheres were placed into a 45×9 mm glass column filled with 3 ml of 50 mM borate buffer, pH 7.4. At the bottom of the column was placed a polyethylene filter to prevent microspheres leakage and both ends were fitted with Teflon tubing. Care was taken to ensure the quantitative transferring of microspheres to the column. Borate buffer was pumped through the column at a flow rate of 800 μl/min by a gradient program able to give increasing concentration of NaCl from 18 to 500 mM. Fractions were collected and analysed for DFT content by UV analysis.

DFT stability after release from microspheres was investigated by analysing the collected fractions by electrophoresis.

Electrophoresis was performed on 3% agarose gels containing 0.5 μg/ml ethidium bromide for 2 h at 25 mV constant current. The relative band migration was determined, after staining the gels with ethidium bromide.

2.6. Cytotoxic activity of cationic microspheres

Cytotoxicity of cationic microspheres was evaluated on human leukemic K562(S) cells. Standard conditions for cell growth were α-medium (GIBCO, Grand Island, NY), 50 mg/l streptomycin, 300 mg/l penicillin, supplemented with 10–15% fetal calf serum (Flow Laboratories, McLean, VA) in 5% CO_2, 80% humidity.

Human leukemic K562(S) cells were treated with different amounts (500, 250, 125, 62.5 or 31.25 μg/ml) of cationic microspheres constituted of $DDAB_{18}$ (30%, w/w) and Eudragit RS. After 6 days of cell culture, the cells were counted by a Fuchs cell counter (Tosenthal, Preciss, France) and the number of cells/ml was compared with the value obtained in untreated cell cultures in order to determine the percentage of surviving cells. Assays were carried out in triplicate and usually counts differed by $< 7\%$.

3. Results and discussion

3.1. Production of Eudragit RS/Eudragit E microparticles

Microparticles were produced using a blend of two polymers, namely the methacrylic polymer Eudragit RS and the cationic methacrylic polymer Eudragit E, by an in-liquid drying process based on evaporation of a volatile solvent from the internal phase of an oil in water emulsion (Esposito et al., 1997).

Microparticle production was performed under the following experimental conditions: a concentration of Eudragit E between 0 and 40% (w/w), a stirring speed between 250 and 1000 rpm and a concentration of the emulsifying agent of 1% (w/w).

From the analysis of the obtained microspheres the following general observations can be gathered: (a) the dimensions of the microparticles are between 21 and 47 μm as function of Eudragit E concentration and stirring speed (see following paragraph); and (b) microparticles are characterized by a porous surface and a rather compact and homogeneous internal structure (Fig. 2).

3.1.1. Influence of experimental parameters on Eudragit RS/Eudragit E microparticles

The effect of some production parameters were investigated on microparticle characteristics. To this aim, firstly microspheres were produced by fixing the stirring speed at 250 rpm and progressively increasing the percentage of Eudragit E, thus varying the number of positive charges present on the particles. It has been found that the microparticle mean diameter is influenced by the amount of Eudragit E employed: in fact the increase in Eudragit E content resulted in a progressive increase in particle diameter, for instance microparticles containing 7.5% Eudragit E have a mean diameter of 33 μm, microparticles containing 15% Eudragit E have a mean diameter of 44 μm, and those containing 30% have a mean diameter of 47 μm (Table 1). The analysis of the microparticle morphology demonstrated that Eudragit E concentration should not exceed 30%, in fact the use of 35% Eudragit E resulted in the production of ellipsoidal microspheres (Fig. 2A) while larger amounts of the cationic polymer led to a worsening of the microparticle shape. Particles in fact became progressively irregular with a pronounced tendency to collapse, forming in addition large aggregates. For this reason the percentage of Eudragit E was fixed at 30% since this concentration enabled microspheres to be obtained with the highest charge density maintaining an acceptable morphology (Fig. 2B).

In a further set of experiments the effect of the stirring speed was investigated on particle characteristics (Table 2). The concentration of Eudragit E was maintained at 30% w/w. As expected, particle dimensions were inversely related to the stirring speed, for instance 250 rpm resulted in microspheres with a mean diameter of 47 μm, while in the case of 1000 rpm microparticle mean

diameter was 21 μm. The morphological characteristics were almost unaffected by the changes of stirring speed, and the microparticle recovery was always over 75% w/w, except for microparticle produced with a stirring speed of 1000 rpm, in which case the microparticle recovery decreased to 64% w/w.

3.1.2. Binding of nucleic acid molecules to Eudragit RS/Eudragit E based microparticles

With the aim of evaluating the capability of cationic microparticles to transport nucleic acid molecules, a series of binding experiments was performed using Defibrotide (DFT) as model compound.

Defibrotide is a single stranded polydeoxyribonucleotide with a molecular weight of 26 200 Da isolated from mammalian lung tissue. DFT was chosen as model nucleic acid molecule since: (a) it mimics the behaviour of low molecular weight nucleic acid antisense oligonucleotides or PCR products for 'decoy' strategies; (b) it is a patented product displaying profibrinolytic and antithrombotic activity currently commercialized in Italy; and finally (c) it is an inexpensive product.

Eudragit RS/E microparticles containing 22.5 or 30% w/w of Eudragit E were suspended in borate buffer containing DFT, using different molar ratios between the positive charges conferred by Eudragit E and the negative charges conferred by DFT (from 6:1 to 24:1, +/−). In Fig. 3 the percentages of association of DFT to cationic microparticles are plotted against the positive to negative charge ratios. From this experiment it is clearly evident that an increase in the proportion of positive charges of the microparticles (obtained increasing the percentage of cationic polymer contained in the microparticles) led to a progressive increase in the association of DFT to the carrier. Unfortunately the association yield of DFT to microspheres was found in all cases to be quite unsatisfactory since the percentage of DFT bounded to microspheres even at the highest positive/negative molar charge ratio never exceeded 20% w/w.

The general characteristics of the microsphere based formulation did not change after addition of DFT, displaying no sign of aggregation, a problem that often occurs with other non-viral delivery systems for nucleic acids.

Table 3
Effect of cationic agent on the production of microparticles composed of Eudragit RS and cationic agents[a]

Cationic agent % (w/w)	Positive charge, μM	Mean diameter, μm	External morphology	Internal morphology	Recovery[b]
CTAB[c]					
9	2.4	28	Spherical shape, porous surface	Lot of hollows	94
13.5	3.6	31	Spherical shape, porous surface	lot of hollows	78
18	4.9	34	Spherical shape, porous surface	Lot of hollows	96
$DDAB_{12}$[c]					
11.2	2.4	27	Spherical shape, wavy surface	Microporous	85
17	3.6	37	Spherical shape, wavy surface	Microporous	83
23	4.9	40	Spherical shape, wavy surface	Microporous	74
$DDAB_{18}$[c]					
15	2.4	29	Irregular shape, microporous surface	Spongy	86
22.5	3.6	32	Almost spherical shape, microporous surface	Spongy	74
30	4.9	41	Spherical shape, porous surface	Spongy	90

[a] Emulsifying agent concentration was 1%, w/w. Stirring speed was always 250 rpm.
[b] Percentage (% w/w) of isolated microparticles with respect to the initial amount of polymer used for microparticles production.
[c] CTAB, cetyltrimethyl-ammonium bromide; $DDAB_{12}$, didodecyl-dimethyl-ammonium bromide; $DDAB_{18}$, dioctodecyl-dimethyl-ammonium bromide.

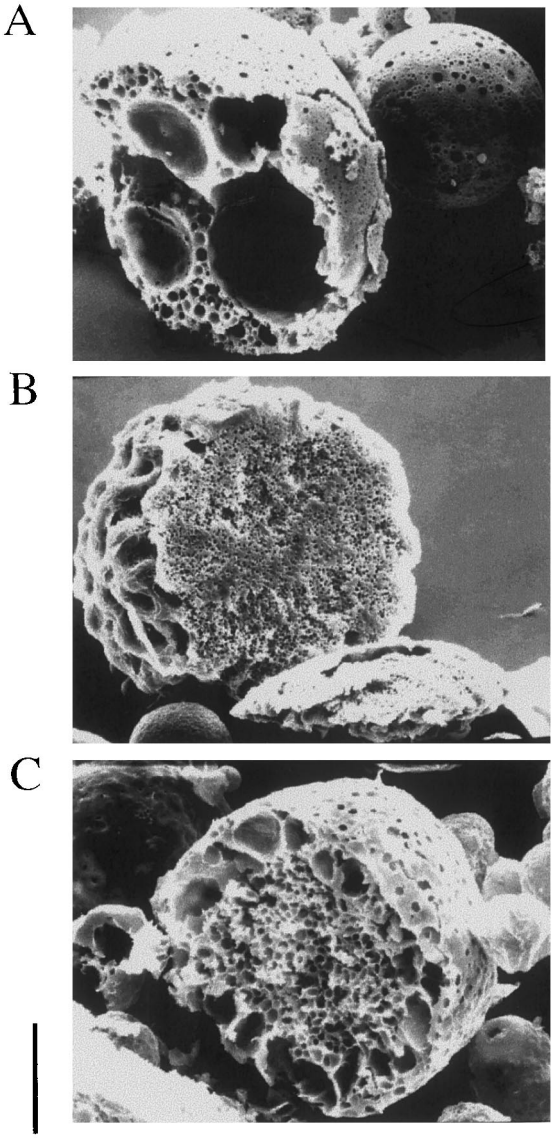

Fig. 4. Scanning electron micrographs of external and internal morphology of microparticles containing 30% CTAB (A), $DDAB_{12}$ (B) or $DDAB_{18}$ (C). Bar corresponds to 60, 50 and 42 μm in panels A, B and C, respectively.

3.2. Production of Eudragit RS/cationic surfactant microparticles

In order to possibly increase the ability of microparticles to bind DFT, cationic microspheres were produced using an alternative approach, namely employing a mixture of Eudragit RS and a cationic surfactant. Initially, for the production of microparticles, three alternative surfactants were used to investigate their performances, namely cethyl-trimethyl-ammonium bromide (CTAB), didodecyl-dimethyl-ammonium bromide ($DDAB_{12}$) or dioctadecyl-dimethyl-ammonium bromide ($DDAB_{18}$). Microparticles were produced using a stirring speed of 250 rpm and an emulsifying agent concentration of 1% w/w.

In Table 3 the concentrations of the cationic surfactants employed are reported. The percentage of cationic agent (w/w) was varied in order to have the same molar amount of positive charges with respect to those present in the Eudragit E based microparticles.

The use of the cationic surfactants resulted in a general reduction of mean diameter when compared to the microspheres produced with the cationic polymer. As found for Eudragit RS/E microspheres, an increase in the percentage of the cationic agent led to an increase in microparticle mean diameters (Table 3). In addition, the type of cationic surfactant employed did not greatly affect the dimension of the particles (Table 3).

All the surfactant based microparticles displayed a spherical shape: those produced with CTAB were characterized by a smooth surface with few small pores with an internal structure characterized by large cavities (Fig. 4A). $DDAB_{12}$ microparticles had a wavy surface with no pores and a microporous internal structure (Fig. 4B). Finally the microparticles produced with $DDAB_{18}$ presented a porous surface and a spongy internal structure (Fig. 4C). In all cases, the produced particles did not present any aggregation phenomenon: microsphere recovery was over 70% (w/w).

3.2.1. Binding of DFT to surfactant based microparticles

Table 4 shows that the use of cationic surfactants as alternative to the cationic polymer Eudragit E significantly improves the binding of DFT to microparticles. For instance, at a positive to negative molar charge ratio of 6 to 1, the percentage of association of DFT to CTAB based microparticles was 63% whilst in the case of Eudragit E, at the same molar charge ratio, the

association was 8.5%. Even better results were obtained with $DDAB_{12}$ and $DDAB_{18}$ based microparticles, which displayed at the same +/− molar charge ratio almost quantitative associations of DFT, namely 91.9 and 88.6% for $DDAB_{12}$ and $DDAB_{18}$, respectively.

3.2.2. Influence of experimental parameters on Eudragit RS/$DDAB_{18}$ microparticles

Due to the high DFT association and the low toxicity of $DDAB_{18}$, the microparticles constituted of Eudragit RS and $DDAB_{18}$ were chosen as model delivery system for a further characterization study. In a previously published paper it has been in fact demonstrated that $DDAB_{18}$ is less toxic with respect to $DDAB_{12}$ when these surfactants are employed in liposomal form (Cortesi et al., 1996). The influence of some experimental parameters was studied on the morphological and dimensional characteristics of the microparticles. In particular, the following parameters were investigated: (a) the stirring speed, (b) the percentage of the emulsifying agent used during the preparation to stabilize the o/w emulsion, and (c) the type of rotor (Tables 5 and 6).

It is interesting to note that in the case of $DDAB_{18}$ microparticles, the progressive increase in the surfactant percentage resulted in an aug-

Table 4
Binding of Defibrotide to cationic microparticles composed of Eudragit RS and $DDAB_{18}$

Molar charge ratio (+/−)	Association[a] of DFT to particles containing $DDAB_{18}$ (3.6 μM)	Association[a] of DFT to particles containing $DDAB_{18}$ (4.9 μM)
2/1	26.4	51.5
4/1	44.2	80.9
6/1	75.0	88.6

[a] Percentage (% w/w) of Defibrotide associated to microparticles with respect to the total amount of Defibrotide used.

Table 5
Effect of stirring speed on the production of microparticles composed of Eudragit RS and $DDAB_{18}$[a]

Stirring speed (rpm)	Mean diameter (μm)	External morphology	Internal morphology	Recovery[b]
250	41	Spherical shape, porous surface	Spongy	90
500	36	Spherical shape, porous surface	Spongy	94
750	27	Spherical shape, porous surface	Spongy	77
1000	23	Spherical shape, porous surface	Spongy	72

[a] Emulsifying agent concentration was 1%, w/w. $DDAB_{18}$ concentration was always 4.9 μM.
[b] Percentage (% w/w) of isolated microparticles with respect to the initial amount of polymer used for microparticles production.

Table 6
Effect of type of rotor on the production of microparticles composed of Eudragit RS and $DDAB_{18}$

Type of rotor[a]	Mean diameter (μm)	External morphology	Internal morphology	Recovery[b]
(a)	36	Spherical shape, porous surface	Spongy	90
(b)	41	Spherical shape, porous surface	Spongy	80
(c)	35	Spherical shape, porous surface	Spongy	82
(d)	50	Spherical shape, porous surface	Spongy	85

[a] (a) a two-blade helical rotor with a diameter of 45 mm, (b) a four-blade helical rotor with a diameter of 50 mm, (c) a three-blade rotor with a diameter of 40 mm, and (d) a clover like rotor with a diameter of 50 mm.
[b] Percentage (% w/w) of isolated microparticles with respect to the initial amount of polymer used for microparticles production. $DDAB_{18}$ concentration was always 4.9 μM.

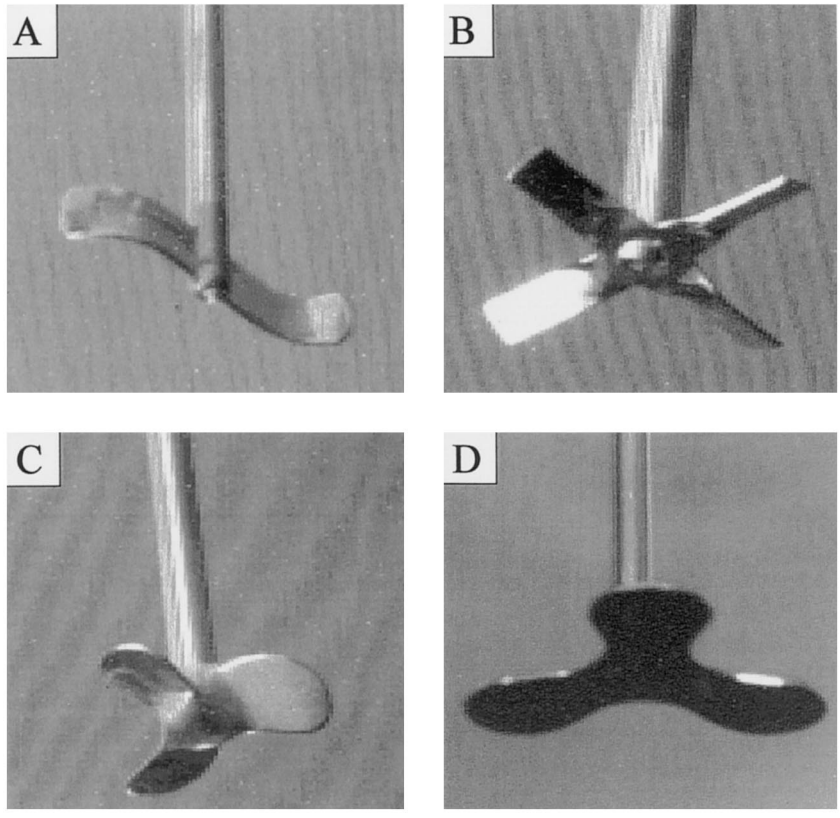

Fig. 5. Rotors employed for cationic microsphere production: (a) two-blade helical rotor (45-mm diameter); (b) four-blade helical rotor (50-mm diameter); (c) three-blade rotor (40-mm diameter) and (d) clover like rotor (50-mm diameter).

Table 7
Effect of percentage of emulsifying agent on the production of microparticles composed of Eudragit RS and $DDAB_{18}$

Percentage of emulsifier	Mean diameter (μm)	External morphology	Internal morphology	Recovery[a]
1	41	Spherical shape, porous surface	Spongy	90
0.5	49	Spherical shape, porous surface	Spongy	90
0.1	55	Irregular shape, porous surface	Spongy	94

[a] Percentage (% w/w) of isolated microparticles with respect to the initial amount of polymer used for microparticles production. $DDAB_{18}$ concentration was always 4.9 μM.

mented sphericity whilst, as previously stated, Eudragit E caused the opposite effect.

The percentage of microparticle recovery was in all cases quite satisfactory, being between 72 and 94%.

The increase in the stirring speed resulted in a decrease in the microsphere dimensions. For instance, the variation of the stirring speed from 250 to 1000 rpm caused almost a halving of the microsphere diameter from 41 to 23 μm (Table 5) without appreciable changes in the morphological characteristics.

The performances of four different rotors were also comparatively investigated (Fig. 5), namely (a) a two-blade helical rotor with a diameter of 45 mm, (b) a four-blade helical rotor with a diameter

of 50 mm, (c) a three-blade rotor with a diameter of 40 mm, and (d) a clover like rotor with a diameter of 50 mm. In particular the use of rotors (b) and (d) allowed microparticles to be obtained with mean diameters of 41 and 50 μm, respectively, while microparticles obtained by rotors (a) and (c) displayed smaller mean diameters (36 and 35 μm, respectively) (Table 6).

With regard to the amount of emulsifying agent employed, the experimental data show that a decrease in the concentration of polyvinyl alcohol in the aqueous phase from 1 to 0.1% w/w resulted in an increase in the mean diameter of the microparticles from 41 to 55 μm (Table 7). It is in fact known that the emulsifying agent is able to decrease the interfacial tension between the organic and the aqueous phase of the o/w emulsion during microparticle production leading to a decrease in the droplet dimensions and a consequent decrease in the final particle dimensions.

The concentration of the emulsifying agent also influenced microparticle morphology. The particles produced with 1 or 0.5% of polyvinyl alcohol were characterized by a spherical shape while a decrease of emulsifying agent to 0.1% resulted in irregular particles.

3.3. Binding of DFT to $DDAB_{18}$ based microparticles

In order to investigate the ability of $DDAB_{18}$ based microparticles to bind DFT, different microparticles were employed, characterized (a) by different concentration of the cationic surfactant and (b) by different dimensions (Tables 8 and 9). Fig. 6 reports the percentages of association plotted against the different molar charge ratios displaying that the percentage of DFT association was directly related to molar charge ratio between microparticles and DFT.

The increase in $DDAB_{18}$ concentration in microparticles as expected led to an increase in DFT association whilst the microparticle dimension was found inversely related to the capability to bind to DFT. This latest result can be explained considering that the smaller microparticles are, the larger is their specific surface thus exposing an increased number of positive charges able to bind to the negatively charged DFT.

Table 8
Association of Defibrotide to cationic microparticles composed of Eudragit RS and cationic agents[a]

Molar charge ratio (+/−)	Association[b] of DFT to particles containing CTAB	Association[b] of DFT to particles containing $DDAB_{12}$	Association[b] of DFT to particles containing $DDAB_{18}$
2/1	22.5	35.1	51.5
4/1	38.5	60.3	80.9
6/1	63.4	91.9	88.6

[a] Surfactant concentration was always 4.9 μM.
[b] Percentage (% w/w) of Defibrotide associated to microparticles with respect to the total amount of Defibrotide used.

Table 9
Association of Defibrotide to cationic microparticles composed of Eudragit RS and $DDAB_{18}$[a]

Molar charge ratio (+/−)	Association[b] of DFT to particles with mean diameter of 23 μm	Association[b] of DFT to particles with mean diameter of 41 μm	Association[b] of DFT to particles with mean diameter of 55 μm
2/1	63.5	52.5	57.8
4/1	92.1	79.6	74.5
6/1	93.0	86.6	82.3

[a] $DDAB_{18}$ concentration was always 4.9 μM.
[b] Percentage (% w/w) of Defibrotide associated to microparticles with respect to the total amount of Defibrotide used.

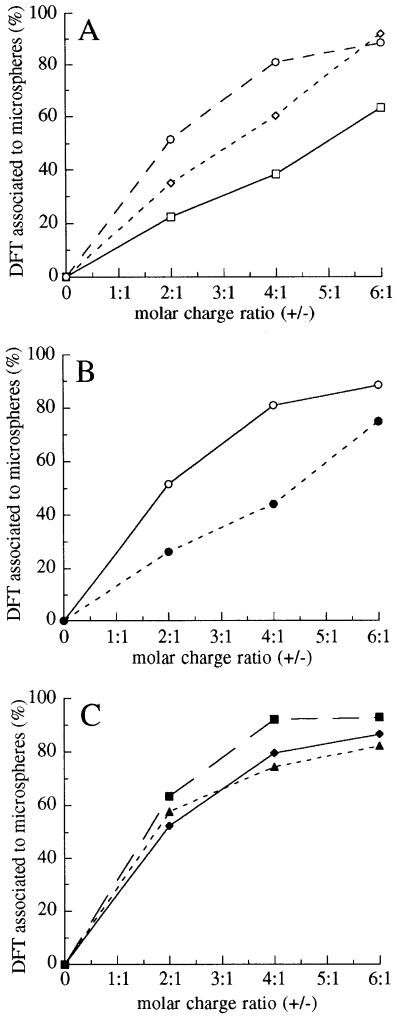

Fig. 6. Percentages of DFT association to: (A) cationic microspheres containing 240 μmol of CTAB (□), $DDAB_{12}$ (◇) and $DDAB_{18}$ (○); (B) cationic microspheres containing 240 (○) or 180 (●) μmol of $DDAB_{18;}$ (C) cationic microspheres containing 240 μmol of $DDAB_{18}$ with mean diameter 23 (□), 41 (◇) and 55 μm (△).

3.4. Release kinetics of DFT from cationic microparticles

The determinations of the release kinetics of DFT from $DDAB_{18}$ containing microparticles were performed by a dynamic flow method using as release medium borate buffer at pH 6.8 containing increasing amount of NaCl from 18 to 500 mM. Fig. 7 shows that the DFT release profile was characterized by a sigmoidal shape: in particular it can be noted that 20% of DFT was released when the molarity of release medium was 75 mM, 50% of DFT was released with a release medium 240 mM, and plateau was reached when NaCl concentration was 400 mM.

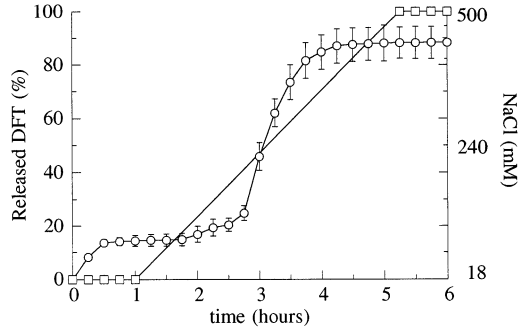

Fig. 7. Release profiles of DFT from cationic microspheres containing $DDAB_{18}$ (○) versus NaCl mM concentration in the release medium (□).

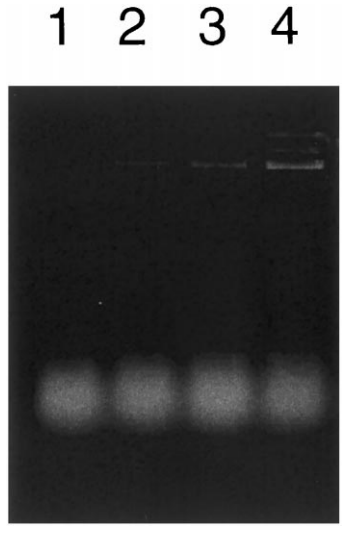

Fig. 8. Agarose gel electrophoresis of DFT containing fractions collected during release experiments from microspheres. Lane 1 refers to DFT solution used as control, and lanes 2, 3, and 4 refer to DFT content in fractions collected after 2, 4 and 6 h of elution.

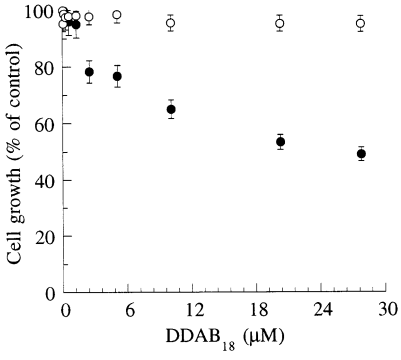

Fig. 9. Cytotoxic activity of $DDAB_{18}$ microparticles (○) and free $DDAB_{18}$ (●) on the cultured human cell line K562. Data represent the % of cell number/ml compared to untreated control K562 cells.

3.5. Stability of DFT

The stability of DFT after association and release from cationic microspheres was evaluated by analysing the DFT present in the release medium by agarose gel electrophoresis. Fig. 8 reports agarose gel electrophoresis of DFT containing fractions after 2, 4 and 6 h. In all cases migration of DFT resulted in a single band similar to that of control indicating that no DFT degradation occurs after 6 h of elution from microspheres.

3.6. Cytotoxic activity of cationic microparticles

An in vitro study was performed for determining the cytotoxic activity of cationic microparticles.

Human leukemic K562(S) cells were treated with the same concentrations, in term of $DDAB_{18}$ molarity, of free or formulated $DDAB_{18}$. After 6 days of cell culture, cells were electronically counted. Fig. 9 reports the cytotoxic activity of $DDAB_{18}$ based microparticles (concentration was between 0 and 500 μg/ml corresponding to a $DDAB_{18}$ concentration between 0 and 250 μm) and free $DDAB_{18}$ at the same concentration. The obtained data demonstrated that $DDAB_{18}$ formulated in microparticles displayed lower cytotoxicity with respect to free $DDAB_{18}$, suggesting that $DDAB_{18}$ based microparticles could be safely used in ex vivo experiments.

4. Concluding remarks

Positively charged microparticles based on Eudragit RS and cationic agents (i.e. a cationic acrylic polymer or cationic surfactants) for the delivery of defibrotide were produced and characterized.

The influence of some experimental parameters on dimensional and morphological characteristics of microparticles was investigated. It was found that (a) the use of cationic surfactant improved microparticle morphology with respect to Eudragit E microparticles, (b) an increase in the cationic agent concentration led to an increase in microparticle mean diameter, (c) an increase in the stirring speed led to a decrease in microparticle mean diameter, and finally (d) an increase in the emulsifying agent concentration led to a decrease in microparticle dimensions and an improvement of their morphology.

The desoxiribonucleotide Defibrotide was associated with positively charged microparticles and its in vitro release kinetics from microparticles were determined. The use of Eudragit RS in association with $DDAB_{18}$ resulted in the highest percentage of recovery (89% with 6:1, $+/-$ charge ratio) and in addition DFT was released in a controlled manner. A study on the in vitro toxicity of cationic microparticles on cultured human cell line K562 was also performed, demonstrating that $DDAB_{18}$ based microparticles display very low cytotoxicity with respect to control untreated cells.

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