Research Article

Comparison on *In Vitro* Characterization of Fucospheres and Chitosan Microspheres Encapsulated Plasmid DNA (pGM-CSF): Formulation Design and Release Characteristics

Ali Demir Sezer[1,2] and Jülide Akbuğa[1]

Abstract. Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a cytokine used in the treatment of serious conditions resulting from chemotherapy and bone marrow transplantation such as neutropenia and aplastic anemia. Despite these effects, GM-CSF has a very short biological half-life, and it requires frequent injection during the treatment. Therefore, the cytokine production is possible in the body with plasmid-encoded GM-CSF (pGM-CSF) coding for cytokine administered to the body. However, the selection of the proper delivery system for the plasmid is important. In this study, two different delivery systems, encapsulated plasmid such as fucoidan–chitosan (fucosphere) and chitosan microspheres, were prepared and the particle physicochemical properties evaluated. Fucospheres and chitosan microspheres size ranges are 151–401 and 376–681 nm. The zeta potential values of the microspheres were changed between 8.3–17.1 mV (fucosphere) and +21.9–28.9 mV (chitosan microspheres). The encapsulation capacity of fucospheres changed between 84.2% and 94.7% depending on the chitosan molecular weight used in the formulation. *In vitro* plasmid DNA release from both delivery systems exhibited slower profiles of approximately 90–140 days. Integrity of released samples was checked by agarose gel electrophoresis, and any additional band was not seen. All formulations were analyzed kinetically. The calculated regression coefficients showed a higher r^2 value with zero-order kinetics. In conclusion, the characterizations of the microspheres can be modulated by changing the formulation variables, and it can be concluded that fucospheres might be a potential carrier system for the controlled delivery of GM-CSF encoding plasmid DNA.

KEY WORDS: chitosan; fucoidan; GM-CSF; microsphere; plasmid.

INTRODUCTION

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a glycoprotein with a molecular weight between 14.5 and 19.5 kDa depending on the degree of glycosylation. GM-CSF, having a broad biological activity, is one of the first identified cytokines (1,2). GM-CSF regulates the viability, proliferation, differentiation, and function of hematopoietic progenitor cells while increasing the viability, function of dendritic cells, the differentiation and growth of dermal Langerhans cells, and antigen-presenting cells' ability to capture foreign antigens (3). GM-CSF is a cytokine used in the treatment of serious conditions resulting from chemotherapy and bone marrow transplantation such as neutropenia and aplastic anemia (4). In addition, its use, alone or in combination with other cytokines, in cancer treatment has been reported to enhance immunity (5). Despite these

pharmacological properties, GM-CSF has a very short biological half-life and therefore requires frequent injection throughout the treatment, causing side effect problems to occur. In addition, very short-term serum stability of cytokine and easy destruction and inactivation of body protein showed the need for investigation of new routes of administration of GM-CSF to the body (6,7). Therefore, in recent years, studies have investigated the efficacy of the systems providing long duration of action in treatment with plasmid DNA coding GM-CSF (8,9). Thus, cytokine production is possible in the body with specific plasmids coding for cytokines administered to the body. However, the most import point here is the proper selection of the system that would transport the plasmid. Cationic polymers and phospholipids are non-viral gene delivery vectors currently investigated. Cationic vectors electrostatically interact with DNA to form a complex, or they are involved in the preparation of particulate systems with DNA (10–12). Chitosan, a polycationic polymer, is the leading polymer used in these systems. However, lower transfection efficiency of the gene delivery systems prepared with chitosan required the modification of the structure or preparation of the particulate system reinforced with chemical agents or ligands.

Chitosan is a linear polymer of [1→4]-linked 2-amino-2-deoxy-β-ᴅ-glucopyranose and 2-acetamido 2-deoxy-β-ᴅ-

[1] Department of Pharmaceutical Biotechnology, Faculty of Pharmacy, Marmara University, 34668, Haydarpaşa, Üsküdar, Istanbul, Turkey.
[2] To whom correspondence should be addressed. (e-mail: adsezer@marmara.edu.tr)

glucopyranose. It is a natural polymer that is derived by *N*-deacetylation of chitin, a component of the shells of shrimp and crab. Chitosan is a non-toxic, biodegradable, natural polysaccharide that is biodegraded by lysozyme to its natural residues of glucosamine and *N*-acetylglucosamine and is a widely used biomaterial with an established safety profile in humans (13). It has been used as a pharmaceutical excipient, drug carrier, mucosal adjuvant, and in FDA-approved hemostatic dressing (14,15). Most importantly, chitosan solution can be formulated under mild aqueous conditions which should not attenuate cytokine bioactivity (16). On the other hand, fucoidan is a sulfated polyfucose polysaccharide and has attracted considerable biotechnological research interest since the discovery that it possessed anticoagulant activity similar to that of heparin and also reported to possess other properties including anti-thrombotic, anti-inflammatory, anti-tumoral, and anti-viral effects (13,17,18). Many of these effects are thought to be due to its interaction with growth factors such as basic fibroblast growth factor and transforming growth factor-β. Fucoidan may therefore be able to modulate growth factor-dependent pathways in the cell biology of tissue repair (19). Although a great number of studies on different pharmacological properties of fucoidan and chitosan are present, there is no information on the fucoidan-based system used in plasmid delivery.

The aim of this study was to prepare a new microsphere system (fucospheres) for encapsulating the plasmid DNA encoding human GM-CSF (hGM-CSF), based on polyion complexation of negatively charged fucoidan with positively charged chitosan, and to evaluate its *in vitro* characterization such as the influence of formulation parameters on the physiochemical properties, encapsulating capacity, and plasmid release compared to fucospheres with chitosan microspheres.

MATERIALS AND METHODS

Materials

Chitosan (M_w 150 kDa, deacetylation degree 93%, 200 mPa.s; M_w 400 kDa, deacetylation degree 87%, 200–400 mPa.s; M_w 600 kDa, deacetylation degree 85%, >400 mPa.s in 1% acetic acid at 20°C) were purchased from Fluka BioChemika (Darmstadt, Germany). Fucoidan (M_w 80 kDa from *Fucus vesiculosus*) used was purchased from Sigma Chemical Co (St. Louis, USA). Sodium sulfate was supported from Carlo Erba (Italy). All other chemicals and solvents used were of pharmaceutical or molecular biology reagent grade.

Plasmid Construction and Isolation

pORF-hGM-CSF plasmid DNA (InvivoGen, San Diego, USA), 3,650-bp size, encodes human GM-CSF and contains elongation factor-1α (EF-1α)/human T cell leukemia virus (HTLV) hybrid promoter which is a composite promoter comprised from EF-1α (1) promoter and 5′ untranslated region of the HTLV; hGM-CSF gene from the ATG to the stop codon; *Escherichia coli* origin of replication; and ampicillin resistance gene as given in Fig. 1 (20).

Plasmid was amplified in *E. coli* GT100, extracted by Birnboim and Doly's modified alkaline lysis method and purified by phenol/chloroform extraction followed by PEG:
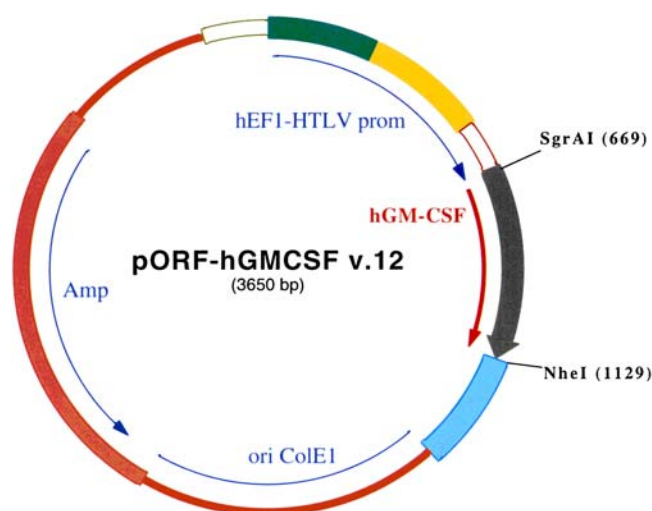

Fig. 1. Schematic diagram of the pDNA encoding pGM-CSF (20)

NaCl extraction and ethanol precipitation (21). The quantity of the purified plasmid DNA was determined spectrophotometrically at 260 and 280 nm (Shimadzu UV-Biospec 1610, Japan), and the quality of the isolated plasmid was confirmed by electrophoresis on a 0.8% (w/w) agarose gel.

Preparation of Plasmid Encapsulated Microspheres

Plasmid encapsulated fucoidan–chitosan microspheres were prepared according to earlier reports of Sezer *et al.* (13,22). The particles were prepared by mixing positively charged chitosan and negatively charged fucoidan using a polyion complexation method. For the preparation of fucospheres, the different amounts of plasmid DNA were mixed with 10 ml of the fucoidan aqueous solution (0.5%, w/v), and this mixture was dropped into 10 ml of the acidic solution (0.25%, w/v) of chitosan (pH 4.5), and this suspension was stirred at 500 rpm at 30 min with a magnetic stirrer (Ika-Werk, Germany) or high shear homogenization (Ika, Euroturrax T20, Germany) at 20,000 rpm at 5 min. Formed microspheres were washed and separated by centrifugation at 15,000g (3K30, Sigma, USA). Then, pellets were freeze-dried (Leybold–Lyovac, Germany). For comparison, chitosan microspheres containing plasmid DNA were prepared as previously described by Berthold *et al.* (23). Briefly, plasmid DNA was added to 10 ml of sodium sulfate solution (20%, w/v), and this mixture was dropped into 10 ml of acidic solution of chitosan (0.25% or 0.50%, w/v) and stirred for different revolutions per minute, then particles were washed and lyophilized. A number of variables were investigated for the optimization of microspheres (Table I).

Agarose Gel Electrophoresis

The integrity of the free and the encapsulated plasmid DNA was analyzed by agarose gel electrophoresis. Amount of plasmid DNA was applied into a 0.8% agarose gel containing Tris-boric acid-EDTA buffer (pH 8.3) and ethidium bromide (0.5 μg/ml) at constant voltage (80 V; Horizontal gel apparatus system, ATTO, Japan). The bands were observed under UV light (Vilber Lourmat, USA), and the conformation of plasmid was checked using the gel documentation system (Kodak

Table I. Composition and Physical Properties of pGM-CSF Encapsulated Chitosan Microspheres and Fucospheres

Codes	Amount of pDNA (μg)	Fucoidan concentration (%)	Chitosan concentration (%)	Chitosan molecular weight (kDa)	Stirring rate (rpm)	Encapsulation capacity (% ± SD)	Before centrifugation		After centrifugation		Production yield (% ± SD)	Zeta potential values (mV ± SD)
							Mean particle size (nm ± SD)	Polydispersity index ± SD	Mean particle size (nm ± SD)	Polydispersity index ± SD		
A1	673	–	0.50	400	500	91.7±0.9	492±32	0.31±0.07	681±23	0.44±0.09	95.6±3.2	28.9±1.2
A2	336	–	0.50	400	500	84.6±2.0	473±21	0.28±0.09	659±26	0.42±0.11	93.1±4.4	27.5±0.8
B1	336	–	0.25	400	500	73.9±1.8	439±28	0.25±0.08	542±17	0.34±0.07	90.6±5.4	21.9±0.5
B2	336	–	0.25	400	20,000	85.3±1.4	255±19	0.23±0.07	376±11	0.35±0.09	84.8±2.6	22.1±0.7
C1	336	0.50	0.25	400	500	85.6±2.0	288±16	0.19±0.06	401±11	0.23±0.12	85.1±3.2	11.4±0.5
C2	336	0.50	0.25	400	20,000	94.0±1.6	132±14	0.12±0.04	177±10	0.19±0.07	80.8±2.5	11.8±0.5
D	673	0.50	0.25	400	20,000	95.2±1.4	145±10	0.13±0.06	180±6	0.20±0.08	81.1±2.7	12.8±0.5
E1	336	0.50	0.25	150	20,000	84.2±1.7	119±8	0.10±0.05	151±6	0.11±0.06	79.6±2.2	8.3±0.6
E2	336	0.50	0.25	600	20,000	94.7±0.8	201±11	0.17±0.06	242±7	0.21±0.12	83.5±3.1	17.1±0.9

Digital Science, DC290 Camera and 1D Image Analysis Software, USA).

Scanning Electron Microscopy

Microspheres were mounted on metal grids using double-sided tape and coated with gold to about 500×10^{-8} cm in thickness using a sputter coater under high vacuum, 0.1 Torr, 1.2 kV, and 50 mA at $25\pm1°C$. Surface morphology of particles was investigated with a scanning electron microscopy (SEM; Joel, JSM-5200, Japan) at 20 kV.

Particle Size Analysis

Particle size distribution (mean diameter and polydispersity index) was determined by photon correlation spectroscopy using a Malvern Nano ZS, model ZEN 3600 (Malvern Instruments, UK). Each particle preparation was analyzed in duplicate with ten readings per particle sample suspended in distilled water. Mean particle size and polydispersity index were calculated for each sample. Mean values of three batches are given in Table I.

Zeta Potential Measurement

The electrophoretic mobility of microspheres was determined using Malvern Nano ZS, model ZEN 3600 (Malvern Instruments). Measurements were carried out at 25°C in phosphate-buffered saline (PBS, pH 7.4, BP), and the results were expressed as a mean of three measurements.

Determination of Entrapment Efficiency

The plasmid DNA content of the microspheres was calculated by the difference between the total amount added in the aqueous solution and the amount of free plasmid in the crude microsphere system. The free plasmid amount in the supernatant was assayed spectrophotometrically at 260 nm ($n=3$).

The entrapment efficiency was calculated by the following equation:

$$\% EE = \frac{A_{\text{initial plasmid DNA}} - A_{\text{free plasmid DNA}}}{A_{\text{initial plasmid DNA}}} \times 100 \quad (1)$$

where $A_{\text{initial plasmid DNA}}$ is the amount of the initial plasmid DNA used in the preparation of the microspheres and $A_{\text{free plasmid DNA}}$ is the amount of free plasmid DNA detected in the supernatant after centrifugation of the aqueous dispersion.

In Vitro Release Studies

The release profiles of plasmid-encoded GM-CSF (pGM-CSF) from the microspheres was determined after incubation of particles in PBS (pH 7.4, BP) in a shaker bath at $37 \pm 0.1°C$ at 100 rpm. Samples were removed and centrifuged for 10 min at $15,000\times g$ (Hettich, Germany), and the supernatant was replaced by fresh medium after each sampling. Optimum centrifugation speed (5,000 rpm) and time (2 min) which could separate the supernatant without causing microsphere aggregation were determined by preliminary studies. The
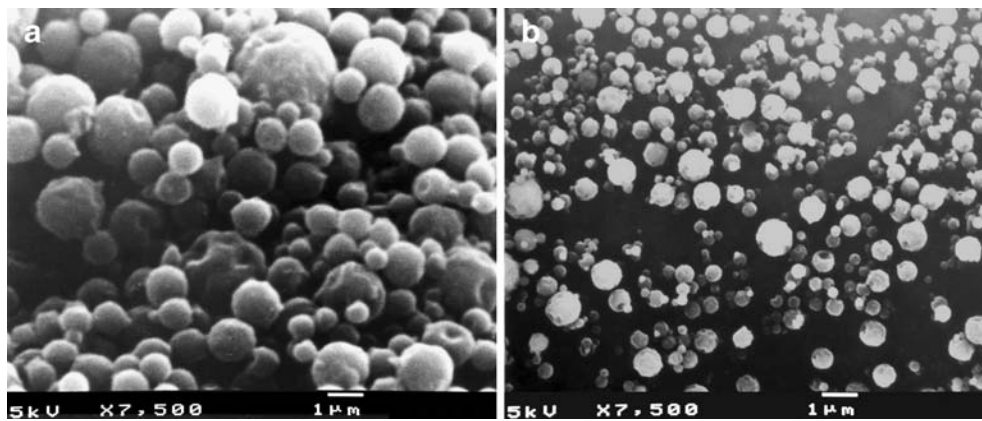
Fig. 2. SEM photographs of (**a**) pGM-CSF encapsulated chitosan microspheres and (**b**) fucospheres

amount of plasmid released was measured spectrophotometrically at 260 nm ($n=3$) (22).

Kinetics of Plasmid DNA Release

The *in vitro* release data of the microspheres were evaluated kinetically by zero-order kinetics, first-order kinetics, Higuchi model, and Hixson–Crowell. The ideal kinetic models were determined using the dissolution kinetics program of Ege University, version 1.0.40.

Statistical Analysis

Results were expressed as mean ± standard deviation. One-way analysis of variance or a *t* test was performed to compare the influence of various parameters. A *p* value <0.05 was considered as representing a significant difference.

RESULTS AND DISCUSSION

Characterization of the Microspheres

In gene therapy, production of a biologically active protein requires that exogenous DNA penetrate the cell membrane to avoid lysosomal degradation and enter the nucleus to undergo transcription (24,25). Non-viral gene

delivery approaches, including those utilizing polycations such as chitosan and polyethylenimine, are commonly utilized due, in part, to the safety concerns associated with viral vectors (12,26). Fucoidan, a unique class of high-molecular-mass sulfated fucans extracted from brown seaweeds, is a biopolymer, and its new microsphere delivery system, named fucosphere, is based on polyion complexation of negatively charged fucoidan with positively charged chitosan (13,22). The observation of the microspheres with SEM confirmed that the particles are well-defined spherical shape having nearly smooth surfaces with a few small pores (Fig. 2). In addition, it appeared that the polymer concentration and chitosan molecular weight had no effect on the surface morphology of microspheres.

The most commonly used separation technique of microspheres from the particle dispersion is centrifugation. The optimization of this process is critical because wrong choice of parameters might lead to complete aggregation and loss of colloidal stability. After preparation, fucospheres had an average diameter between 119 and 288 nm, and chitosan nanoparticles varied between 255 and 492 nm with a narrow size distribution (polydispersity index). After drying, whereas the particle size of the fucospheres ranged between 151 and 401 nm, the size of chitosan microspheres varied between 376 and 681 nm (Table I). The microspheres with smallest particle size were obtained with increasing the preparation stirring rate and using the lowest polymer amount in the formulation.
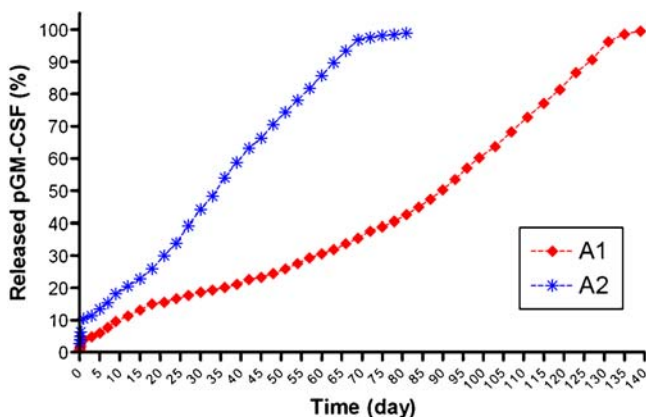

Fig. 3. Effect of initial plasmid amount on release behavior of pGM-CSF encapsulated chitosan microspheres
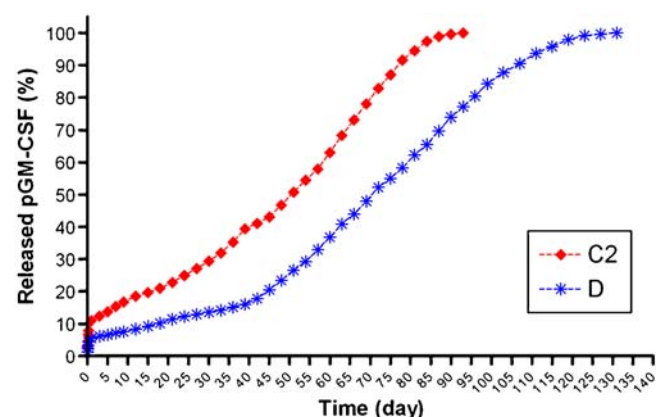

Fig. 4. Effect of initial plasmid amount on release behavior of pGM-CSF encapsulated fucospheres
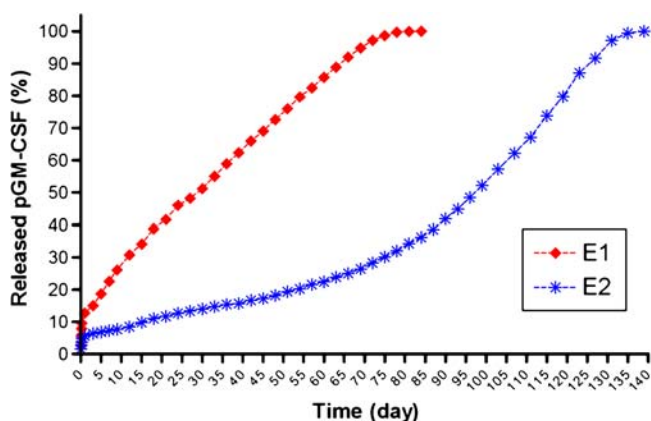
Fig. 5. Effect of chitosan molecular weight on release behavior of pGM-CSF encapsulated fucospheres

Table II. Release Kinetic Values (r^2) of pGM-CSF Encapsulated Chitosan Microspheres and Fucospheres

Codes	Zero order (r^2)	First order (r^2)	Higuchi (r^2)	Hixon Crowel (r^2)
A1	0.9496	0.6006	0.8405	0.7772
A2	0.9945	0.8417	0.9489	0.9390
B1	0.9913	0.8328	0.9856	0.9500
B2	0.9951	0.8453	0.9583	0.9360
C1	0.9912	0.7018	0.9331	0.8670
C2	0.9731	0.6850	0.8802	0.8434
D1	0.9564	0.7292	0.8450	0.8595
E1	0.9824	0.7688	0.9824	0.9348
E2	0.8795	0.5199	0.7390	0.6935

By increasing the concentration of chitosan in the formulation, particle size of fucospheres was also increased, whereas high stirring rate reduced the particle size (Table I). Particle size was decreased approximately 50% by increasing the preparation stirring rate of fucospheres (Table I). On the other hand, molecular weight of chitosan affected the mean particle size of fucospheres ($p<0.05$). Mean particle size of formulation E1 containing chitosan with molecular weight of 150 kDa (151 nm) was found to be smaller than formulation E2 (242 nm) prepared with 600 kDa chitosan ($p<0.05$). The results were in accordance with the literature (27,28). The encapsulated plasmid DNA amount did not affect the particle size of whole formulations ($p>0.05$). The particle size distribution (polydispersity index) of fucospheres (0.11–0.23) was found to be narrower than the chitosan microspheres (0.34–0.44). Increasing of chitosan concentration ($p<0.05$) and molecular weight ($p>0.05$) widened the size distribution of particles.

The zeta potential values of fucospheres were between +8.3 and +17.1 mV, and chitosan microspheres changed between 21.9 and 28.9 mV (Table I). An increase in chitosan concentration enhanced the surface charge of formulations (A2, 27.5 mV and B1, 21.9 mV). Positive zeta potential value was recorded for the microsphere formulations when the amount of positively charged chitosan was higher than that of negatively charged fucoidan in microsphere structure. This indicates that the outer surface of the fucospheres consisted of chitosan (29,30). It was suggested that as a result of interaction between protonated amine groups of chitosan and non-protonated sulfate groups of fucoidan, excess of chitosan chain forms the outer layer of the fucospheres, and chitosan and fucoidan complex constitutes a hydrophobic core. The amount of protonated amine groups of chitosan on the outer surface of fucospheres significantly changed the surface charge of microspheres, as seen with chitosan–glycyrrhetic acid nanoparticles (31) and chitosan–heparin complexes (32).

Plasmid DNA encapsulation efficiency of fucospheres enhanced upon increasing the molecular weight of chitosan used, and burst release decreased in the same conditions. Indeed, the encapsulation capacity ranged from 73.9% to 91.7% for chitosan microspheres and 84.2% to 95.2% for fucospheres. However, chitosan concentration has a marked influence on the encapsulation capacity of the chitosan particles; increasing the chitosan concentration from 0.25% to 0.50% led to an increase in microsphere encapsulation capacity from 73.9% to 84.6%. Similar results were obtained for fucospheres prepared with different molecular weights of chitosan in the formulation. Plasmid DNA encapsulation efficiency of fucospheres fabricated with low-molecular-weight chitosan was found to be lower than those prepared
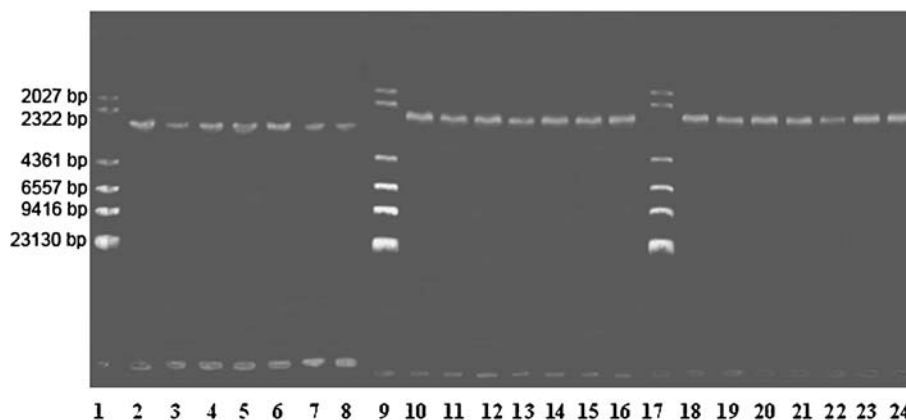

Fig. 6. Agarose gel photographs of pDNA after isolation and pGM-CSF after released from microspheres. *Lines 1, 9,* and *17* are *Hin*dIII digested λ DNA marker, *lines 2, 10,* and *18* are pGM-CSF after isolation. *Lanes 3–8* and *11–13* are pDNA released from microspheres (A1–E2) after 30 days. *Lanes 14–16* and *19–24* are pDNA released from microspheres (A1–E2) after 60 days

with medium- and high-molecular-weight chitosans ($p<0.05$), but there was no significant difference on encapsulation efficiencies of fucospheres prepared with medium- and high-molecular-weight chitosans ($p>0.05$). On the other hand, preparation stirring rate affected the encapsulation efficiency of whole particles ($p<0.05$). It was also obtained that the encapsulation capacity was increased significantly when the amount of pGM-CSF increased two times ($p<0.05$; Table I). A thin polymeric wall is this formed around the droplets immediately after mixing of the two phases (internal and external), leading to a rather high encapsulation efficiency for fucospheres and chitosan microspheres. The fine layer of polymer precipitated at the interface acts then as a barrier against the plasmid leakage and the shear stress of the subsequent stirring (32,33). This behavior was confirmed by the high pGM-CSF encapsulation efficiency obtained.

In vitro release profiles of pGM-CSF loaded microspheres in PBS (pH 7.4) medium are given in Figs. 3, 4, and 5. An initial burst effect was detected and then slow release observed at constant rate from fucospheres and chitosan particles. Burst release might be related to plasmid DNA adsorbed onto the surface of particles. Plasmid DNA release was limited with amount of pGM-CSF as well as chitosan molecular weight and concentration (Figs. 3 and 5; $p<0.05$). The plasmid release from microspheres slowed down significantly when the amount of pGM-CSF increased in the formulation. Release patterns of both microsphere systems were significantly different, and the plasmid DNA release from fucospheres was slower than that of chitosan particles (Figs. 3 and 4; $p<0.05$). These results are similar with the results of Yoksan and Akashi (34) and Khatri *et al.* (35). The molecular weight of chitosan affected the plasmid release from microspheres due to the increasing amine groups of the polymer with increased molecular weight and length of chitosan chain in the particle structure. Consequently, increase of amine groups can raise binding of polymers to electronegative groups of plasmid DNA (Fig. 5; $p<0.05$) (36). On the other hand, high-molecular-weight chitosans with high deacetylation degree are soluble up to pH 6.5. Glucosamine units of chitosan shows high density of amine groups and requires pH values higher than 6.5 to be insoluble with slow swelling (37). However, similar release profiles were observed in a previous study with microspheres in which the protein encapsulated (22). The *in vitro* release showed that the low-molecular-weight chitosan-based fucospheres tended to weaken bonding and release DNA. The influence of chitosan molecular weight on formation of the microspheres could be explained by the chain entanglement effect (38). When comparing the low-molecular-weight chitosan with medium and high ones used in fucosphere formulations, condensed plasmid DNA was less efficient at the same N/P ratio as a result of its shorter polymer chain and lower entanglement capability. For this reason, low-molecular-weight chitosan-based microspheres with a smaller size and lower association efficiency were formed and showed rapid release than the other fucospheres (Fig. 5; $p<0.05$). The relationship between chitosan molecular weight and the binding ability of chitosan to DNA in fucospheres and chitosan microspheres was further confirmed by the gel retardation assay. It was shown that the particles prepared with medium- and high-molecular-weight chitosan were dissociated, and the plasmid DNA migrated out of the loading well, whereas there was no dissociation for the particles. Our findings

are supported by the literatures (39,40). The preparation stirring rate did not affect the pGM-CSF release from fucospheres and chitosan microspheres (figure not given; $p>0.05$).

As seen on the release profiles in Figs. 4 and 5, pGM-CSF released from the fucospheres showed a three-phasic behavior. In the first 24 h, nearly 10% of the plasmid rapidly released from the particles. In the second phase, approximately 20–50% of the plasmid DNA released from the microspheres at constant speed. At the final step, microspheres degraded after the completion of swelling process and plasmid release was faster than the second phase ($p<0.05$).

In order to determine the structural integrity of the pDNA released from fucospheres and chitosan microspheres, agarose gel electrophoresis was applied after purification of the samples. As seen in Fig. 6, supercoiled topological form of released pDNA between 2,322 and 4,361 bp was observed on the gel electrophoresis. In order to compare the isolated pGM-CSF and release samples, any additional band on the gels was not seen. All formulations were analyzed for the plasmid release kinetically. The calculated regression coefficients showed a higher r^2 value with zero-order kinetics (between 0.8795 and 0.9951; Table II).

CONCLUSIONS

The polyion complexation method was developed in order to formulate plasmid GM-CSF containing microparticles prepared with fucoidan and chitosan as a new plasmid carrier microsphere system for gene therapy. Plasmid DNA was successfully encapsulated into fucospheres with changes in some formulation parameters. The described formulation and process allowed achieving high encapsulation efficiency and low burst effect compared with chitosan microspheres. The structure of the microspheres is related to chitosan's molecular weight, polymer concentration, and the preparation stirring rate. The highest encapsulation capacity and the slowest plasmid DNA released from microspheres were obtained with using the highest amount of plasmid DNA (D) and chitosan molecular weight (E2). It was observed that the plasmid release kinetic fit to zero-order release profiles.

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