Biomaterials 30 (2009) 4815–4823

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

A chitosan hydrogel delivery system for osteosarcoma gene therapy with pigment epithelium-derived factor combined with chemotherapy

Hang T. Ta [a], Crispin R. Dass [b,c,*], Ian Larson [d], Peter F.M. Choong [b,c,e], Dave E. Dunstan [a]

[a] Department of Chemical and Biomolecular Engineering, University of Melbourne, Victoria 3010, Australia
[b] Department of Orthopaedics, University of Melbourne, Melbourne, Victoria 3065, Australia
[c] Department of Surgery, St Vincent's Hospital, Melbourne, Victoria 3065, Australia
[d] Monash Institute of Pharmaceutical Sciences, Monash University, Victoria 3052, Australia
[e] Sarcoma Service, Peter MacCallum Cancer Centre, Victoria 3002, Australia

ARTICLE INFO

Article history:
Received 16 March 2009
Accepted 18 May 2009
Available online 7 June 2009

Keywords:
Chitosan
Orthophosphate
Drug delivery
Hydrogel
Osteosarcoma
Cancer

ABSTRACT

Osteosarcoma (OS) is the most common type of malignant bone cancer, and the sixth most common type of cancer in children and young adults. Currently, gene therapy is being evaluated as a novel method for OS treatment. Here we report on an in situ gelling chitosan-based hydrogel system that sustains the release of a potential anti-cancer gene (pigment epithelium-derived factor) to the tumor site. A significant reduction of the primary OS in a clinically relevant orthotopic model was measured. The combination of plasmid treatment and chemotherapy together with the use of this delivery system led to the highest suppression of tumor growth without side effects. The results obtained from this study demonstrate the potential application of a biodegradable hydrogel technology as an anti-cancer drug delivery system for successful chemo-gene therapy.

1. Introduction

OS is the most common primary solid tumor of bone in children and young adults [1,2], comprising about 20% of primary bone sarcomas [3], and represents the second highest cause of cancer-related death in this age group [4]. Currently, the primary treatments of this disease include chemotherapy and surgery. Chemotherapy drugs can be administered both before and after surgery. Contemporary chemotherapy is normally the intravenous or oral administration of different chemotherapeutic agents [5,6]. However, most chemotherapeutics carry the risk of both short-term and long-term toxic effects. In addition, despite improved aggressive treatment comprising of multi-agent chemotherapy and surgical resection, approximately 30–40% of all patients still develop lung metastasis which is the major cause of death from this disease [4,7]. Since the current regimes used to treat OS have failed to eradicate the systemic spread of OS, alternative treatment such as gene therapy which should exclude the problems faced with current cytotoxic agents such as drug resistance and systemic toxicity has attracted significant research interest.

The study of chronological and spatial patterns of OS growth and invasion of local tissue structures including growth plate cartilage in bone has revealed that this relatively avascular structure acts as a natural barrier to the progression of this tumor [2]. This corresponded with the growth plate layers with high expression of the most potent endogenous anti-angiogenic factor, pigment epithelium-derived factor (PEDF). Therefore, PEDF may have potential therapeutic applications in OS, by improving the local control of tumor growth and increasing the potential for limb-sparing surgery, as well as reducing the risk of metastatic spread in this disease. Ek et al. have demonstrated the therapeutic potential of PEDF against OS using two clinically relevant orthotopic models of OS and a set of cell-based assays relevant to OS proliferation, apoptosis, collagen-1 adhesion, differentiation, angiogenesis and metastasis [7].

The delivery of foreign DNA into host cells has been accomplished by non-viral vectors such as liposomes and DNA–polymeric complexes [8], and by viral vectors such as retroviral and adeno-associated viral vectors [9]. However, the efficiency of non-viral vectors is very low and they are rapidly inactivated in the presence of serum. On the other hand, viral vectors have provided high transduction efficiency but faced serious safety problems with some clinical deaths reported [9]. Recently, PEDF plasmid (pPEDF) has been encapsulated in chitosan microparticles as a delivery vehicle for OS treatment [10], though proper efficacy was not determined.

* Corresponding author. Department of Orthopaedics, St Vincent's Hospital, Melbourne, Victoria 3065, Australia.
E-mail address: crispin.dass@svhm.org.au (C.R. Dass).

0142-9612/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2009.05.035

In the previous study, we have developed a thermal and pH responsive chitosan in situ gelling system using low concentrations of an inorganic phosphate salt, dipotassium orthophosphate (DPO) as the gelling agent [11]. We have also demonstrated the sustained releasing properties of Chi/DPO hydrogels which are liquid at low or room temperature but gel at body temperature (37 °C). The successful application of this biodegradable and biocompatible Chi/DPO hydrogel in the delivery of Doxorubicin (Dox), a small anti-tumor molecule, for the treatment of OS in a clinically relevant orthotopic mouse model has been also reported [12]. In this paper, a further application of Chi/DPO technology in delivering pPEDF, a large bioactive molecule, in OS treatment was presented. The same clinically relevant metastatic mouse model of OS [4] was employed in this study. The dual-pronged regime for OS treatment provided by the combination of chemotherapy and plasmid treatment was discussed.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan from crab shells (~120 kDa) with deacetylation degree between 75% and 85% was obtained from Fluka BioChemika (Switzerland). Dipotassium phosphate (K_2HPO_4) (DPO) and acetic acid (CH_3COOH) were purchased from Ajax Finechem (Australia). Doxorubicin was obtained from Sigma–Aldrich, Australia. PEDF plasmid was obtained as previously described [10]. Briefly, PEDF DNA insert was cloned into pcDNA3.1-his-myc(−)A vector (Invitrogen) for transfection. The plasmids expressing PEDF (pPEDF) and the pcDNA3.1 empty vectors (pVector or pVec) were amplified using TOP10 bacterial cells (Invitrogen, Australia). The human SaOS-2 OS cell line attained from the American Type Culture Collection (ATCC) was cultured in α-MEM (Invitrogen, Australia) supplemented with 10% fetal calf serum (FCS, Invitrogen, giving complete medium, CM) in a humidified 5% CO_2 atmosphere. Cells were used within 20 passages. A seeding cell population of exponentially-growing cells greater than 95% viability was used for all assays. Female 5-week-old Balb/c nude mice (Animal Resource Center, Perth, Australia) were used for orthotopic injection of SaOS-2 cells. Mice were housed and maintained at the BioResources Centre (St Vincent's Hospital, Australia) as previous described [13]. All animal experimentations were approved by the St Vincent's Health Animal Ethics Committee.

2.2. Preparation of hydrogel solution

Low M_w chitosan solutions were prepared by dissolving chitosan flakes in 0.1 M acetic acid overnight at room temperature under constant stirring. The resultant solutions were then filtered through 100 μm pore sized filters and stored under refrigeration, at 4 °C. Chitosan solutions of different concentrations were heated at 85 °C for different numbers of hours to reduce their viscosity. DPO solutions were prepared with distilled water and stored at 4 °C. Appropriate amounts of cold DPO solution (4 °C) were added into cold chitosan solution (4 °C) and mixed manually at room temperature until homogeneous.

2.3. Rheology analysis

Rheological measurement was performed using a Carri-Med CSL^2 100 controlled stress rheometer with cone (4 cm diameter, 1.59° angle) and plate geometry. All the measurements were performed in oscillation mode at a fixed frequency of 1 Hz and a strain of 1%, which is well within the measured linear viscoelastic region. The temperature evolution of G' and G'' moduli were measured with change in temperature at a rate of 1 °C/min. The time evolution of G' and G'' were measured at constant temperature of 37 °C. The gelation temperature and the gelation time were taken as the temperature and the time at which G' and G'' were equivalent in value [14–16].

2.4. In vitro release study

Appropriate amounts of pPEDF were added to DPO solutions and mixed thoroughly. After being kept cold at 4 °C, the resultant DPO/pPEDF solution was mixed manually with an appropriate amount of cold chitosan solution (4 °C) until homogeneous. Five different Chi/DPO-pPEDF formulations were prepared as detailed in Table 1.

20 μl of the resultant Chi/DPO solution containing pPEDF was pipetted into a 1.5 ml Eppendorf tube which was incubated at 37 °C for 1 h at which temperature the solution gelled. The formed gels occupied the bottom of the tubes and were exposed to the release buffer by the same surface areas (~19.6 mm^2). 1 ml of PBS pH 7.2 (GIBCO, Invitrogen, Australia) was pipetted into each tube. Controls included tubes containing blank hydrogel. The tubes were then placed in a shaking incubator (Ratek, Australia) maintained at 37 °C under gentle shaking at 100 rpm.

Table 1
Chi/DPO formulations for pPEDF delivery.

Formulation	Components					
	Low M_w chitosan solution			K_2HPO_4 solution		pPEDF[a] (μL)
	Concentration (w/w%)	Heat-treating time (h)	Volume (μL)	Concentration (w/w%)	Volume (μL)	
Chi/DPO6-pPEDF	2	0	900	50	60	68.8
Chi/DPO7-pPEDF	2	0	900	50	50	68.0
Chi/DPO8-pPEDF	2.5	9	900	25	70	69.6
Chi/DPO9-pPEDF	2.5	9	900	28	70	69.6
Chi/DPO10-pPEDF	2.5	9	900	22	100	71.2

[a] pPEDF stock solution is 7.5 g/l. Final concentration of pPEDF in formulations is 0.5 mg/ml Chi/DPO.

At the predetermined time intervals, 900 μL of the release buffer was sampled and stored at −20 °C for further analysis. Before sampling, the Eppendorf tubes were quickly centrifuged at 700 × *g* for 1 min to make sure all damaged gels staying at the bottom of the tubes and not being sampled. Subsequently, 900 μl of fresh buffer was added to the tubes in order to maintain constant volume of release medium. pPEDF released from the hydrogel formulations was exponentially amplified by Polymerase Chain Reaction (see Section 2.5) and then quantified by Agarose Gel Electrophoresis (Section 2.5). The density of ethidium bromide stainings of amplified pPEDF standards was plotted versus pPEDF standard concentration. The amount of the released pPEDF was calculated based on the standard curve obtained from this plot.

The *in vitro* release of pPEDF was also conducted in the presence of lysozyme (Invitrogen, Australia) at concentration of 8 μg/ml, which is similar to the enzyme concentration found in the human body. Released pPEDF solutions were concentrated by isopropanol method (see Section 2.6) for using in the cell-based assays.

2.5. Polymerase chain reaction and agarose gel electrophoresis

The PCR Master (Roche Diagnostics, Australia) kit was utilized according to manufacturer's protocol. Forty cycles were run at $T_{annealing}$ of 63 °C, $T_{elongation}$ of 72 °C and $T_{denaturation}$ of 94 °C. The PCR was carried in small reaction tubes (0.2 ml) (Roche Diagnostics), containing a reaction volume of 44 μl, that were inserted into a thermal cycler, PCR Eppendorf Mastercycler (Roche Diagnostics). PCR products were run on a 2% agarose gel in Tris–acetate–EDTA buffer at 90 V for 1 h. The gel was visualized and analyzed using a BioRad Gel-Doc system with Quantity One software (Australia).

2.6. Plasmid DNA extraction

The released plasmid pPEDF solutions were concentrated by using isopropanol precipitation method. The triplicate release solutions for each collection day were combined. Briefly, one volume of cold isopropanol was added into the same volume of pPEDF release solution in a microcentrifuge tube. Since the release buffer (PBS, pH 7.2 – GIBCO, Invitrogen, Australia) contained monovalent cations, there was no need to add any salts to facilitate precipitation. The mixture was mixed gently and placed in fridge at −20 °C for 30 min. The tubes were then centrifuged at 4 °C and 13,200 *g* for 30 min. The supernatant was carefully discarded and the DNA pellet was resuspended in 450 μL of 70% ethanol. After 5 min, the tube was centrifuged at 4 °C and 13,200 *g* for 20 min. Supernatant was removed and discarded. The pellet was air-dried for 1 h then suspended in 30 μL TE (Tris 10 mM, EDTA 1 mM) buffer, pH 8.0.

The extraction efficiency of the plasmids was 61 ± 11% as evaluated using PCR and agarose gel electrophoresis methods. Briefly, 5 μl of the concentrated pDNA solution underwent PCR as described in Section 2.5 but 15 PCR cycles were used instead of 40. The PCR product was quantitated using agarose electrophoresis method as described in Section 2.5.

2.7. Visualization of pDNA in the gel matrix

Chitosan hydrogel containing pPEDF (Chi/DPO7-pPEDF) was immersed in the solution of 10 μg/ml ethidium bromide (EB) for 10 min in dark. Chi/DPO7-pPEDF was then observed under Nikon fluorescence microscope with TR filter and images were taken.

2.8. Proliferation/viability assay

SaOS-2 cells were seeded at 100,000 cells/well in a 24-well plate. After 1 day, cells were transfected with Lipofectamine 2000 (Invitrogen, Melbourne, Australia) containing either released pPEDF or released pVector according to the manufacturer's instruction. Briefly, an appropriate volume of concentrated pDNA solution (obtained from the DNA extraction described in Section 2.6) containing 90 ng pDNA was diluted in Opti-MEM I Reduced Serum Medium (Invitrogen, Australia). Lipofectamine was diluted (1:25) in Opti-MEM I medium (Invitrogen, Australia) and

Table 2
Mice treatment cohorts.

Group	Injection per mouse	Site of injection
Control (no treatment)	100 μL of 0.9% normal saline	Intraperitoneal
pPEDF	2 × 20 μL of pPEDF solution in 0.9% normal saline at 20 μg pPEDF/mouse	Peritumoral
pVec	2 × 20 μL of pVec solution in 0.9% normal saline at 20 μg pVec/mouse	Peritumoral
Chi/DPO7-pVec	2 × 20 μL of Chi/DPO7-pVec solution at 20 μg pVec/mouse	Peritumoral
Chi/DPO7-pPEDF	2 × 20 μL of Chi/DPO7-pPEDF solution at 20 μg pPEDF/mouse	Peritumoral
Combination (combo): Chi/DPO3-Dox + Chi/DPO7-pPEDF	2 × 20 μL of Chi/DPO3-Dox at 5 mg Dox/kg mouse; 2 × 20 μL of Chi/DPO7-pPEDF solution at 20 μg pPEDF/mouse	Peritumoral

Chi/DPO3-Dox is the hydrogel formulation containing Doxorubicin (Dox) [12].

incubated for 5 min at room temperature. Subsequently, 50 μl diluted pDNA was combined with 50 μl diluted Lipofectamine. The resultant solution was incubated for 20 min at room temperature. 100 μl of pDNA–Lipofectamine complexes was then added to each well containing cells and medium without antibiotics. Cells were then incubated at 37 °C for 1 day. Wells were washed with PBS and cells were trypsinized. Cells were enumerated using trypan blue and a hemocytometer.

2.9. Collagen-1 adhesion assay

96-Well plates were coated with 0.2 g/l collagen-1 [4]. Either pVector- or pPEDF-transfected cells (obtained from the trypsinization of cells in the viability assay – Section 2.8) were seeded at a density of 4000 cells/well and incubated at 37 °C for 1 h. Wells were washed twice with PBS (pH 7.2) to remove non-adherent cells. The remaining cells were stained with Hoechst 33258 (Sigma–Aldrich, Australia) and enumerated at 100× magnification using the Nikon microscope/SPOT Advanced software under a UV filter.

2.10. In vivo efficacy study

Mice were anaesthetised with ketamine (100 mg/kg) and xylazine (10 mg/kg). 10 μL of 50% matrigel containing 20,000 of SaOS-2 cells was intratibially injected into each mouse as described previously [4,13,17]. 3 weeks later, mice were randomized into 6 groups with 5 mice per group and administered treatments. Details of these treatment groups are summarized in Table 2. Mice were checked 3 times a week. At the termination of the study (12 days later), tumors were measured using digital calipers and mice legs were X-rayed [10]. Mice were sacrificed and heart, lungs, tumor, skin, and limbs were removed and fixed in 4% para-formaldehyde. After rinsing with PBS, number of lung macrometastases were counted. All tissues were embedded in paraffin for histological analysis.

2.11. Histology

The limbs were decalcified and all tissues were embedded in paraffin for histological analysis according to standard conditions [7]. All tissues were sectioned at 4 μm and stained with hematoxylin and eosin as before. Sections were observed with a Nikon Eclipse TE2000-U microscope (Nikon, Australia) and photographed with SPOT Advanced software (SciTech, USA).

2.12. Statistical analysis

Data were analyzed for statistical significant using Student's *t*-test (2-tailed). A *P*-value ≤ 0.05 was considered significant.

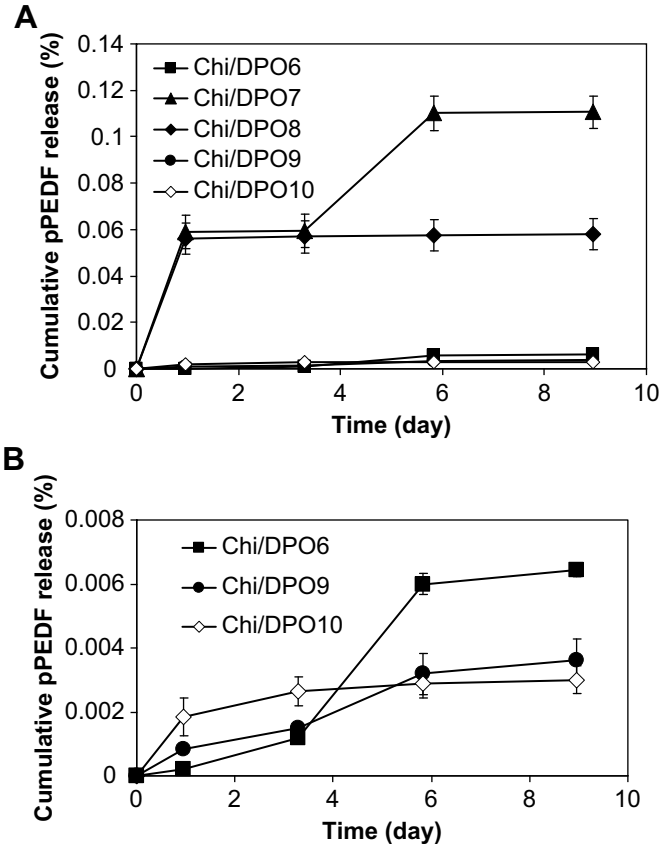

Fig. 1. *In vitro* release of pPEDF from Chi/DPO formulations with the same pPEDF loading (0.5 mg plasmid/ml Chi/DPO) in PBS (pH 7.2) at 37 °C ($n = 3$). (A) All formulations, (B) slow releasing formulations.

3. Results

3.1. Characterization of Chi/DPO solutions

Five different Chi/DPO formulations were characterized and tested in an *in vitro* release study to figure out the most suitable formulation for delivery of pPEDF. They were prepared by varying chitosan concentration, orthophosphate concentration and drug loading parameters (Table 1).

3.1.1. Rheological properties

These five formulations were rheologically characterized to define their gelation temperatures, gelation time at both room temperature (20 °C) and physiological temperature (37 °C), and gel strength at 37 °C. The pH of each formulation was also determined. The results are summarized in Table 3.

3.1.2. In vitro release properties

The *in vitro* release of bioactive ingredient pPEDF from these five formulations was studied for 9 days. It was found that Chi/DPO

Table 3
Properties of Chi/DPO formulations.

Formulation	Gelation temperature (°C)	Gelation time at 20 °C (min)	Gelation time at 37 °C (min)	G' at 37 °C (Pa)	pH at 20 °C
Chi/DPO6	28.2	7.1	Immediate	258.7	7.51
Chi/DPO7	30.6	30.1	0.1	171.1	7.42
Chi/DPO8	33	52.0	4.4	209.6	7.26
Chi/DPO9	32.4	45.0	1.7	248.5	7.33
Chi/DPO10	28.9	14.3	0.7	480.6	7.43

Table 4
Release characteristics of Chi/DPO-pPEDF formulations.

Formulation	Cumulative release after 9 days (%)	Cumulative release after 9 days (ng)	Average release rate (ng/day)
Chi/DPO6	0.006	1.2	0.13
Chi/DPO7	0.11	22	2.4
Chi/DPO8	0.06	12	1.33
Chi/DPO9	0.004	0.8	0.09
Chi/DPO10	0.003	0.6	0.07

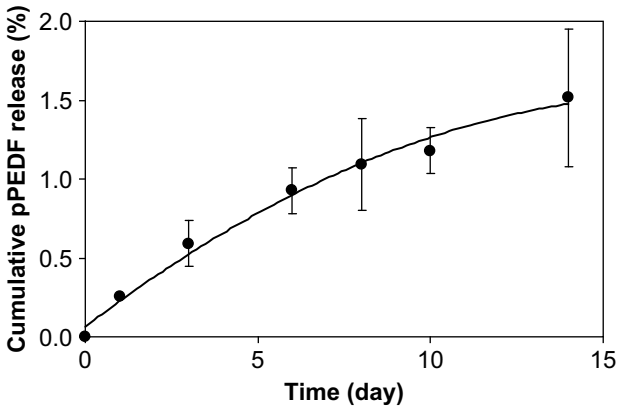

Fig. 2. *In vitro* release of pPEDF from Chi/DPO7 formulation (2.5 mg pPEDF/ml Chi/DPO7) in PBS (pH 7.2) containing lysozyme at 37 °C (lysozyme: 8 μg/ml – similar to the enzyme concentration found in human serum 27) ($n = 3$).

released pPEDF in a sustained manner (Fig. 1A, B) however the release rates were very slow. No initial burst effect was observed in the release of plasmid, a large bio-molecule. Details of the release rate and the cumulative release percentage after 9 days for each formulation were summarized in Table 4. It was observed that within 9 days of the experiment, the size and the shape of the hydrogels did not change significantly.

Based on the pH, the gelation temperature, the gelation time at room temperature and at 37 °C, and the *in vitro* release rate, Chi/DPO7 emerged as the optimum system for delivery of pPEDF. To avoid complication during formulation and delivery, the pH of Chi/DPO needs to be around physiological pH (7.4). Furthermore, the Chi/DPO solution should gel in a reasonable time, not too short to allow enough time for injection, but not too long to prevent abundant drug leaking from an incompletely-formed hydrogel. In addition, the hydrogel matrix should release drugs or active therapeutic agents in a sustained manner. Since an extremely large molecule such as plasmid diffused very slowly out of the gel matrix, the optimum formulation should be the one able to release plasmid relatively fast. Therefore, Chi/DPO7, the formulation releasing pPEDF the quickest, was selected. In addition, Chi/DPO7 owned appropriate gelation temperature (30.6 °C), good gelation time at 37 °C (0.1 min – gel nearly immediately after injection), great stability at 20 °C (30.1 min – long enough for preparation and injection procedure), and suitable pH (7.42 – exactly physiological pH).

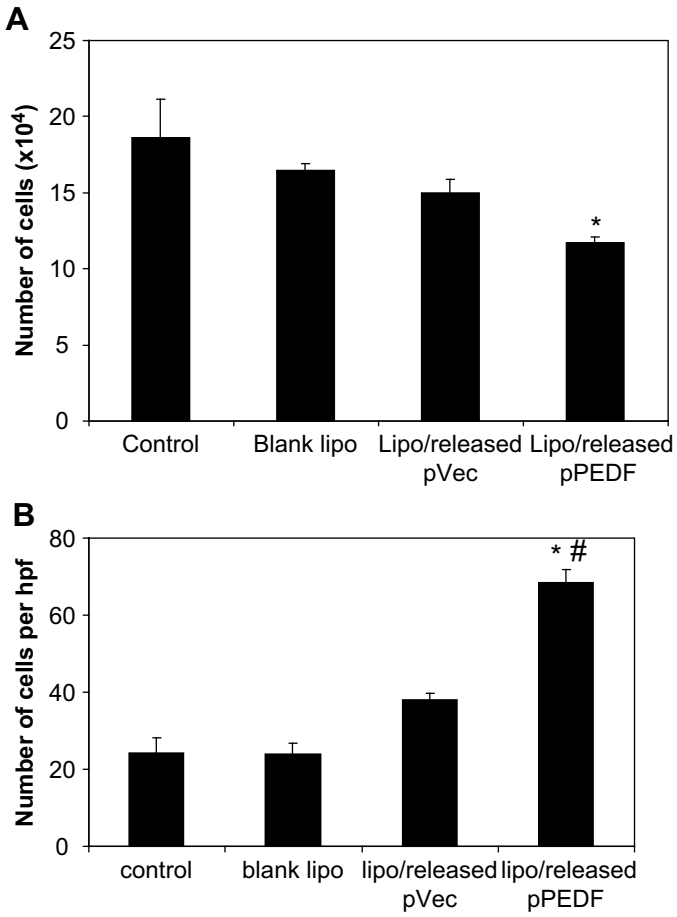

Fig. 4. Cell-based assays. (A) Proliferation assay results graph – inhibition effect of pPEDF released from Chi/DPO7 on SaOS-2 cell proliferation after 1 day of incubation. $^{*}P < 0.05$ versus lipo/released pVec group. (B) Adhesion assay results. $^{*}P < 0.005$ versus control group. $\#P < 0.005$ versus lipo/released pVec group. Control group: no lipo and lipo complexes added.

The *in vitro* release of pPEDF from Chi/DPO7 in PBS buffer containing lysozyme was also performed and is presented in Fig. 2. In the presence of the enzyme, both the initial burst and the release rate after the burst were dramatically increased. After 2 weeks of experiment, around 1.5% (300 ng) of entrapped pPEDF was released. The average release rate was 0.11%/day or 21.4 ng/day

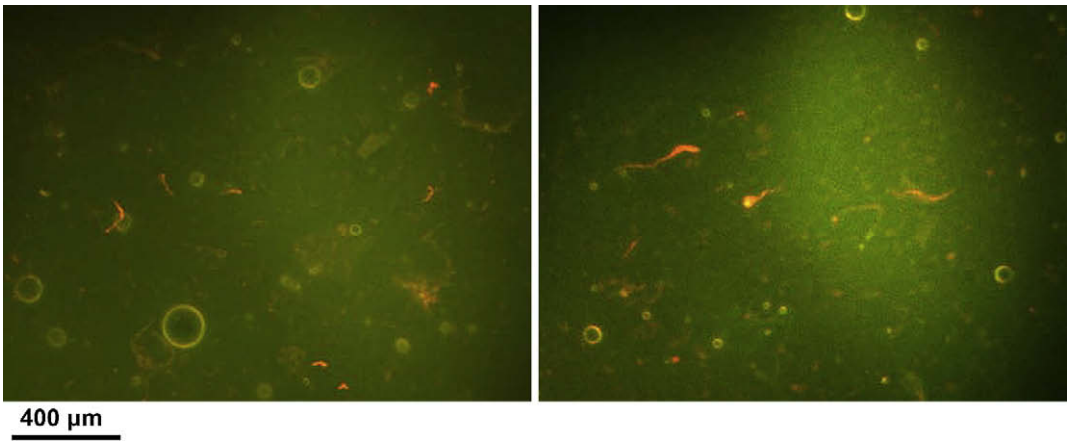

Fig. 3. Color fluorescence images of Chi/DPO-pPEDF. Orange-red fluorescence indicated the presence of plasmid in Chi/DPO hydrogel.

much higher than those in the absence of lysozyme. In the presence of lysozyme, the gels were partially degraded.

3.2. Distribution of pPEDF in the gel matrix

As shown in Fig. 3, pPEDF exhibited orange-red fluorescence after soaking in an ethidium bromide solution, providing a direct visual evidence for the distribution and structure of plasmid in hydrogel. Small blocks or aggregates of pPEDF molecules were regularly distributed throughout the hydrogel matrix.

3.3. Therapeutic activities of pPEDF released from Chi/DPO7 hydrogel

SaOS-2, a human OS cell line, was employed to investigate the *in vitro* activities of pPEDF released from Chi/DPO7 hydrogel matrix. It was known that pPEDF is able to inhibit the proliferation of cancerous cells and capable to increase the adhesion of these cells to collagen-1. In this study, the released pPEDF was transfected to OS cells using Lipofectamine (lipo). It was found that the complexes of lipo/released pPEDF caused noticeable inhibition effect on OS cell proliferation (Fig. 4A). Approximately 37% of cells were dead, compared to the control. It was also noted that lipo and the complexes of lipo/released pVec exhibited slight toxicity on the viability of SaOS-2 cells. As can be seen in Fig. 4B, the incorporation of the released pPEDF on lipo increased the cellular adhesion to collagen-1 coating of the wells by approximately 2.8 times compared to the control group, and by approximately 1.8 times compared to the lipo/released pVec group. These findings demonstrate the preserved functionality of the released pPEDF.

3.4. In vivo efficacy study

3.4.1. In vivo inhibition of primary tumor growth

Fig. 5 reveals the ability of Chi/DPO7-pPEDF to inhibit primary tumor growth at the tibia site. It shows a significant difference between tumor growth in the untreated (control) group and the Chi/DPO7-pPEDF-injected group (Fig. 5A and B). Chi/DPO7-pPEDF

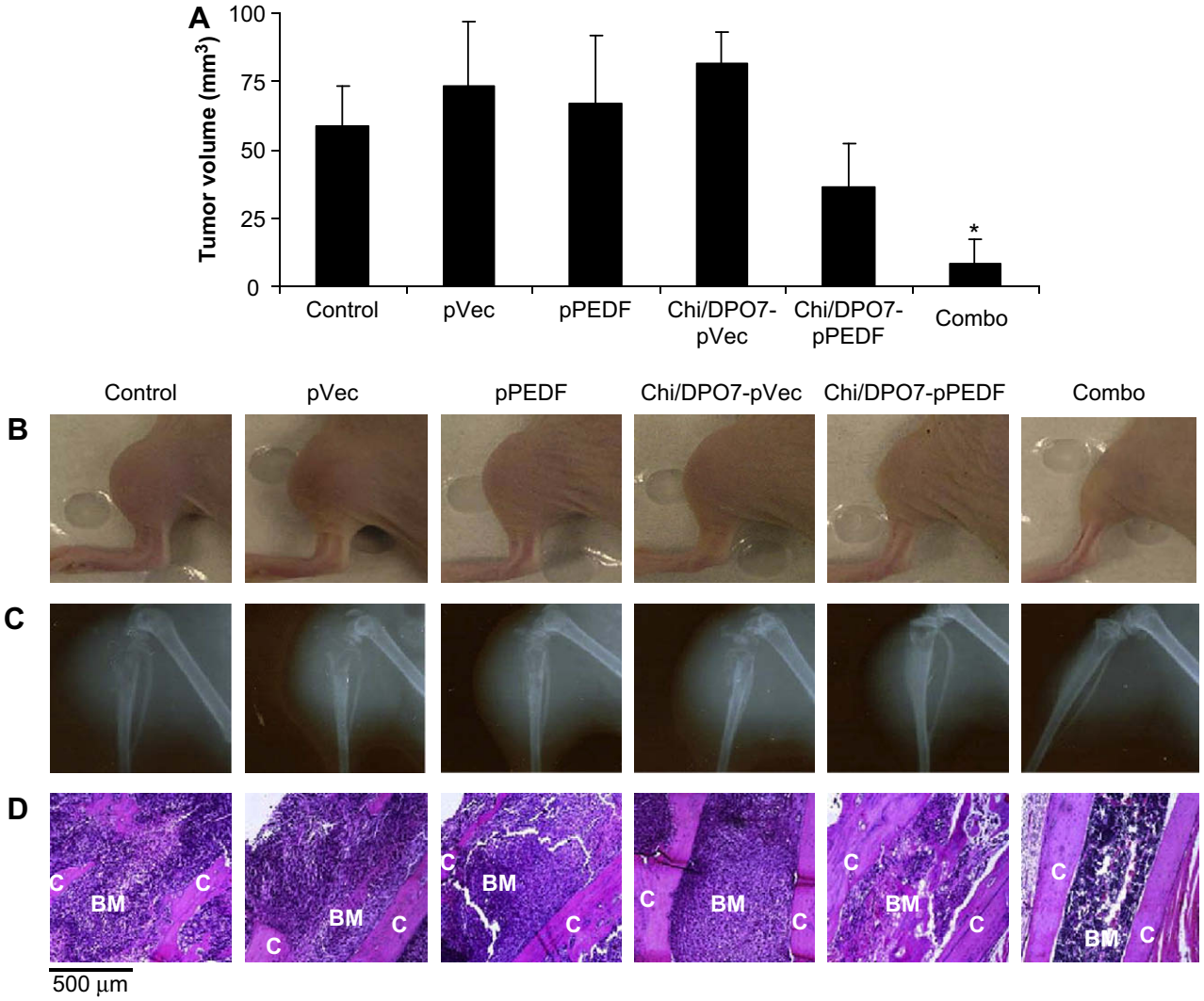

Fig. 5. Inhibitory effect of Chi/DPO7-pPEDF on *in vivo* tumor growth in mice after 12 days post-treatment. (A) Tumor-growth inhibition graph, $^{*}P < 0.05$ versus control and pPEDF group. (B) Primary tumors, showing significantly large tumors in samples from control, pVector, pPEDF and Chi/DPO7-pVector groups; small tumor in sample from Chi/DPO7-pPEDF group; and lack of tumor growth in sample from the combination group. (C) X-ray images, indicating severe bone lysis in samples from pVector, pPEDF and Chi/DPO7-pVector groups, while only mild osteolysis observed in sample from Chi/DPO7-pPEDF group and normal bone cortex in sample from the combination group. (D) Histological analysis showing strong tumor growth severely degrading bone cortex and invading bone marrow in samples from control, pVector, pPEDF and Chi/DPO7-pVector groups; less bone degradation in sample from Chi/DPO7-pPEDF group; and normal bone morphology in sample from the combination group (BM: bone marrow, C: cortex) (magnification 100×).

reduced the tumor volumes by approximately 37% compared to the control group. Interestingly, the combination of Chi/DPO3-Dox and Chi/DPO7-pPEDF (combination treatment group) mostly eliminated the primary tumors. Some mice in this treatment group did not show any tumors at the end of the study. X-ray images revealed lesser evidence of bone degradation (osteolysis) in the groups of Chi/DPO7-pPEDF and normal bone cortex was found in mice belonging to the combination group (Fig. 5C). Histology analysis also confirmed less osteolysis in Chi/DPO7-pPEDF group and no bone degradation in the group of combination treatment (with bone marrow preservation clearly noted) while significant osteolysis occurred in the other cohorts of mice (Fig. 5D).

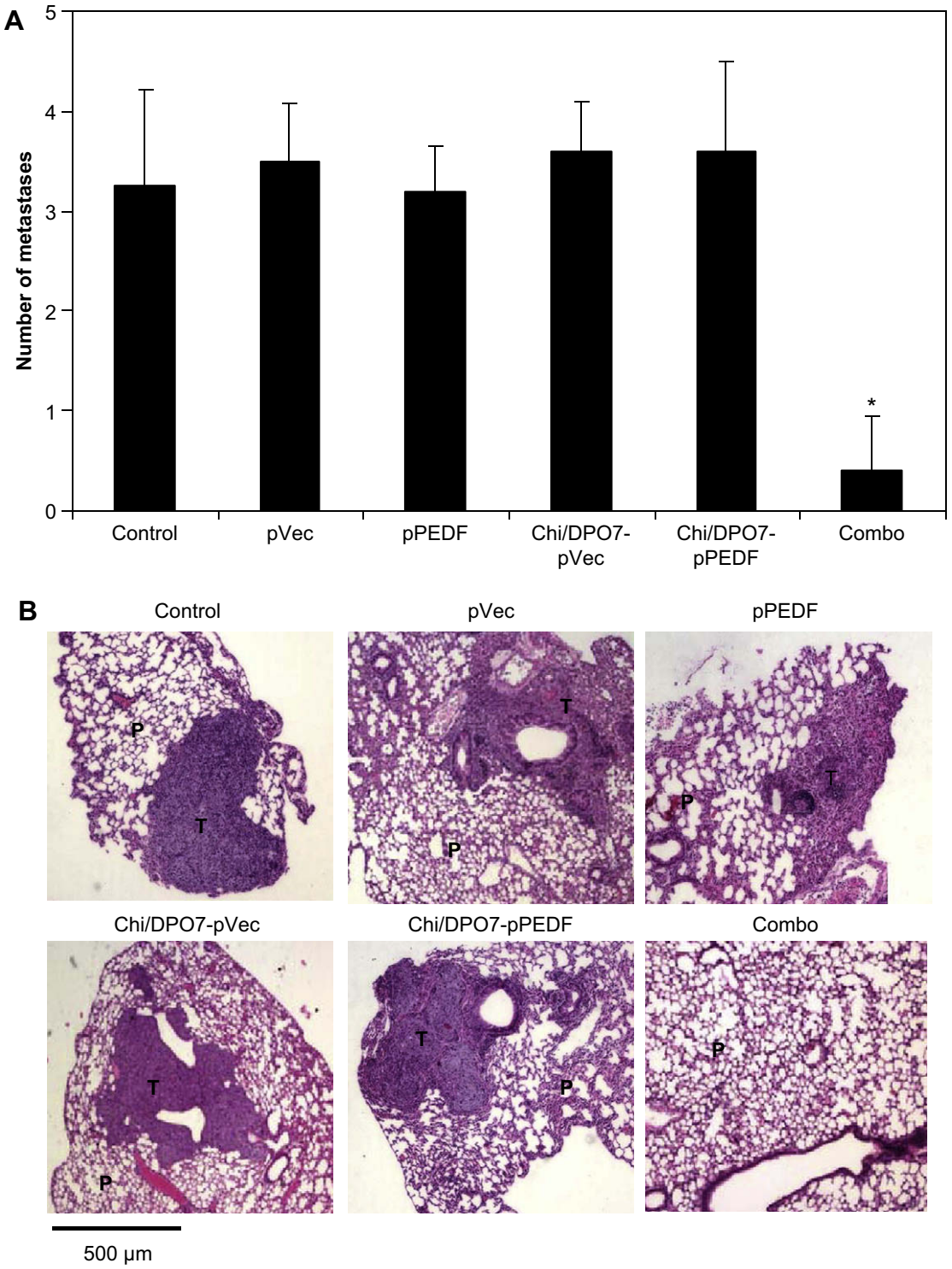

Fig. 6. Inhibition of lung macrometastasis in mice after 12 days post-treatment. (A) Lung macrometastasis inhibition graph, $^{*}P < 0.005$ versus control group. The number of macrometastases was enumerated on the surface of all lobes of each lung against a white background. (B) Histological analysis of lung metastasis (P: parenchyma, T: tumor) (magnification 40×).

3.4.2. In vivo inhibition of secondary tumor growth

Despite the demonstrated potent activity of Chi/DPO7-pPEDF at the primary bone tumor site, the incorporation of plasmid in Chi/DPO hydrogel did not show any positive effect on the development of pulmonary (lung) metastases as can be seen in Fig. 6. However, in the combination treatment group, the number of metastases significantly reduced by approximately 8-fold, indicating a synergic effect due to the dual-pronged therapy. In some mice belonging to this group, metastases were absent altogether. Histological examination of dissected lungs revealed smaller or nil metastasis noted in the combination treatment group.

4. Discussion

PEDF, a widely expressed 50 kDa secreted glycoprotein is a member of the serine protease inhibitor (serpin) family, although it does not inhibit proteases [18]. It has been identified as one of the most potent endogenous inhibitors of angiogenesis. It induces endothelial cell apoptosis through the Fas/FasL death pathway and decreases the expression of important pro-angiogenic factors such as VEGF [19–21]. It also promotes cell differentiation and influences cell proliferation by regulating the cell cycle [22] and inducing apoptosis [23]. In an endothelial cell migration assay performed by Dawson et al., it was found that PEDF was more potent than any of the other known angiogenesis inhibitors. It was more than twice as potent as angiostatin and more than 7 times as potent as endostatin [19].

The release patterns of pPEDF from Chi/DPO formulations were not influenced by both chitosan and orthophosphate concentrations in formulation. They were factors affecting the release of proteins entrapped in Chi/DPO hydrogels as presented in our previous study [11]. In the case of pPEDF, hydrogel strength was the key factor influencing pPEDF release profiles. No initial burst effect was observed in the release of this plasmid, a large bio-molecule.

After 9 days of the study, Chi/DPO7, the weakest hydrogel (171.1 Pa – Table 3) released the highest percentage of entrapped pPEDF (0.11% or 22 ng). Chi/DPO8 with hydrogel strength of 209.6 Pa came second in term of fast release. It released 0.06% or 12 ng of pPEDF throughout the whole experiment. Chi/DPO6, Chi/DPO9 and Chi/DPO10 came next following the order of increasing hydrogel strength and the order of decreasing release rate. There was only 0.003% or 0.07 ng of the entrapped pPEDF released from Chi/DPO10, the strongest formulation.

These results can be explained by the molecular weight and the electrostatic charge of the loaded plasmid. Since pPEDF is an extremely large molecule with M_w of 4.4×10^6 g/mol, it difficultly diffused out of the gel matrix, resulting in greatly slow release rates. In addition, at neutral pH, pPEDF is negatively charged. Since chitosan is a polycationic macromolecule [24], it is likely to form electrostatic interaction with pPEDF, thus limiting the release of pPEDF. Therefore, the release of large molecule such as plasmid was not governed by the diffusion mechanism.

Although the degradation of Chi/DPO hydrogels and the release of plasmid from these formulations were slow in *in vitro* conditions, it was thought that these rates would be much faster *in vivo* due to the presence of various types of cells and a range of degradative enzymes. It is well known that, in human serum, *N*-acetylated chitosan is mainly depolymerized enzymatically by lysozyme [25]. The enzyme degrades the polysaccharide by hydrolyzing the glycosidic bonds present in the chemical structure. It contains a hexameric binding site [26], and hexasaccharide sequences containing 3–4 or more acetylated units contribute mainly to the initial degradation rate of *N*-acetylated chitosan [27]. Human lysozyme is a 15 kDa single chain protein of 123 amino acids [28]. It is present in many human cells and tissues, as well as in soluble form in various body fluids such as serum, umbilical cord serum, urine, cerebrospinal and amniotic fluid, and most secretion fluids including gastric juice, bile, saliva, tears, seminal fluid, and milk [29,30]. The enzyme was also reported to be synthesized during active phagocytosis after nerve injury [31]. Thus lysozyme released from phagocytic cells including macrophages and neutrophils can contribute to the degradation of chitosan and chitin. The specific degradation by lysozyme of chitosan and chitin can be beneficial in tissue engineering and drug delivery applications.

To further mimic the physiological conditions in the human body and to enhance the release rate of pPEDF from Chi/DPO7, lysozyme was added to the release medium at a concentration of 8 mg/l or 8 μg/ml, which is similar to the concentration of lysozyme in human blood serum [32]. As predicted, the release rate was much faster in the presence of lysozyme. The release pattern was approximately linear with a lesser rate of release occurring post-linear phase. The morphology of Chi/DPO7 was observed under SEM showing a heterogeneously porous structure. Chi/DPO7 possessed the pores with their sizes in the range between 10 and 50 μm (data not shown). The outer pores were greater in size compared to the inner pores, which could explain the retarding release rate of plasmid in the later stage. The smaller pores meant more bonds or more connecting fibers existing in the hydrogel matrix, which resulted in the slower degradation rate and then the slower release rate. The results obtained from this study demonstrated that degradation of hydrogel matrix was the main release mechanism for plasmid PEDF.

An experiment was performed to investigate the distribution and the structure of plasmid incorporated in Chi/DPO hydrogel by soaking the Chi/DPO7-pPEDF briefly in an aqueous ethidium bromide solution. Ethidium bromide (EB) is a DNA-intercalating dye used widely to visualize double-stranded DNA in applications such as agarose gel electrophoresis [33]. The fluorescence intensity of EB increases dramatically upon intercalation into double-stranded DNA. Specifically, it yields a high level of fluorescence in the presence of double-stranded DNA and does not stain or label single-stranded DNA well. The fluorescence of EB increases 21-fold upon binding to double-stranded RNA and 25-fold on binding double-stranded DNA so that destaining the background is not necessary with a low stain concentration (10 μg/ml) [34]. In this study, orange-red fluorescent aggregates of pPEDF were observed throughout the hydrogel. The fact that the plasmids existed in the form of several small aggregates in the Chi/DPO matrix may be due to the high viscosity of chitosan solution and the way used to mix chitosan and salt components (manual mixing), which hindered the even distribution of plasmid molecules. Although it is not possible to conclude more details about the specific structure or conformation of the entrapped pPEDF from this basic experiment, the fluorescence observed in Fig. 3 suggests that the plasmid remained substantially double-stranded and not degraded upon incorporation into the hydrogel.

To further investigate the conformation and the bioactivity/functionality of released pPEDF, experiments with SaOS-2, a human OS cell line, were carried out. To be expressed within a cell, the delivered plasmid has to be in its intact functional form. It was found that open circular DNA was transcriptionally competent and could yield high levels of gene expression in transfected cells [35–37].

To evaluate the transcriptional viability of the plasmid released from Chi/DPO hydrogel, these plasmids were transfected into SaOS-2 cells using the commercially available lipid-based transfection reagent Lipofectamine 2000. The efficient transfection of most cell lines *in vitro* generally requires the use of an auxiliary gene transfer agent such as Lipofectamine or a cationic polymer-based delivery system to enhance cellular uptake [38]. *In vivo*, however, the naked or noncomplexed plasmid DNA is well transfected into cells and tissues [36,37]. The transfected cells were then taken into proliferation/viability and collagen-1 adhesion studies. Since PEDF overexpression

significantly reduced OS tumor cell proliferation and increased the adhesion of tumor cells to collagen type-1 [7], the effect of the released pPEDF on SaOS-2 proliferation and adhesion properties can determine the extent of the functionality of plasmids released from Chi/DPO matrix.

The ability of SaOS-2 cells to propagate in the bone cavity and metastasize to the lungs may partly be due to the rapid growth of these cells and the decreased level of adhesion of these cells in poly-L-lysine and more importantly, collagen-1, the most abundant extracellular protein in human bone [4]. Thus *in vivo*, SaOS-2 would readily dissociate from the primary site of bone and intravasate into the bloodstream. Therefore, enhancing the adhesion of SaOS-2 cells to collagen-1 is very important to prevent metastases.

The results obtained from the cell-based assays have confirmed the bio-functionality of released pPEDF. These findings also demonstrated that pPEDF maintained its intact conformation after being released out of the hydrogel matrix. It was noted that the incorporation of pVec in this liposome resulted in the slight effects on SaOS-2 proliferation and its adhesion property. The reason for this phenomenon is yet unknown, but could be due to the transfection procedure. In any case, pPEDF effects were always more pronounced than with pVec.

The efficacy of pPEDF incorporated in Chi/DPO7 hydrogel was investigated *in vivo* using an orthotopic metastatic OS model developed by Dass et al. [4]. The local delivery of pPEDF from the formulation injected peritumorally and its anti-tumor activity were demonstrated via the investigation of tumor growth. Chi/DPO7-pPEDF reduced the tumor volumes by approximately 37% compared to the control group. However, the same did not hold for the free pPEDF-injected groups, which did not exhibit any growth inhibition. Over the 12 days of experiment, the control tumors were $58.44 \pm 14.93\ mm^3$ in size and the free pPEDF-injected tumors had size at $67.12 \pm 24.70\ mm^3$ while the Chi/DPO7-pPEDF treated tumors were only $36.51 \pm 15.99\ mm^3$. The difference was statistically significant ($P < 0.05$) between the Chi/DPO7-pPEDF group and both the control and the free pPEDF groups. Free pVec and Chi/DPO7-pVec did not show any effect on the growth of OS tumors. These results indicate that Chi/DPO7-pPEDF treatment had significantly better ability to delay tumor growth, compared to the free pPEDF treatment with the same pPEDF dose.

The no effect of Chi/DPO7-pPEDF on the development of metastasis may be explained by the slow release of pPEDF from the hydrogel matrix. The *in vivo* efficacy study started after 3 weeks post-inoculation of SaOS-2 cells in mice tibia to induce OS. At this time point, tumors were palpable and became aggressive that could cause a drop in animal body weights [4]. Due to the slow release of pPEDF, it was unable to prevent the fast-growing spread of cancerous cells to the bloodstream, leading to pulmonary metastases. However, as more plasmids were released over time due to the degradation of the hydrogel matrix, they had potency to inhibit the growth of primary tumors as described above.

This paper has reported the application of Chi/DPO hydrogels in the localized delivery of an anti-tumor plasmid pPEDF for OS treatment. Chi/DPO7-pPEDF exhibited potent ability to hamper the growth of primary tumors but did not show any positive effects on the development of pulmonary metastases due to the slow release of pPEDF. In the previous study, the successful application of Chi/DPO hydrogels in the localized delivery of a chemotherapeutic agent Dox for OS treatment was also reported [12]. Chi/DPO3-Dox was able to dramatically inhibit the growth of both primary tumor at the mouse tibia site and the secondary tumor in lungs. However both these treatments could not completely eliminate the presence of tumors. To enhance the tumor-growth inhibition effect, the combined administration of both Chi/DPO3-Dox and Chi/DPO7-pPEDF into mice was performed.

As expected, the combination of Chi/DPO3-Dox and Chi/DPO7-pPEDF (combination treatment) showed the highest inhibition effect on the growth and development of both primary and secondary OS. In the previous paper, it was reported that Chi/DPO3-Dox reduced the tumor volumes by approximately 50% [12]. In this paper, Chi/DPO7-pPEDF was reported to reduce the tumor volumes by 37%, compared to control groups. Interestingly, the combination treatment mostly eliminated the primary tumors. Chi/DPO7-pPEDF, Chi/DPO3-Dox and the combination treatment exhibited inhibition potency in an increasing order. Similarly, the potency of Chi/DPO7-pPEDF, Chi/DPO3-Dox and combination therapy on the inhibition of pulmonary metastases also appeared in an increasing order. The number of pulmonary (lung) metastases decreased approximately 1.5 fold with the Chi/DPO3-Dox group [12]. The inhibition of metastasis was not observed in the group of Chi/DPO7-pPEDF. The number of metastases significantly reduced by approximately 8-fold in the combination treatment group, indicating a synergic effect due to the dual-pronged therapy.

5. Conclusion

The potential application of Chi/DPO hydrogel DDS for OS treatment has been demonstrated again in this paper. pPEDF, a potential anti-tumor bio-agent was successfully released from this DDS in a sustained manner with its functionality preserved. The release of PEDF plasmid was mainly governed by the degradation of Chi/DPO hydrogel. The incorporation of pPEDF in Chi/DPO significantly inhibited the growth of OS and osteolysis. However, due to the slow release of pPEDF from the hydrogel matrix, lung metastases were not reduced or prevented. The peritumoral administration of free pPEDF at the tibia site did not show any inhibition effects on the development of both primary and secondary tumors. This may be due to the rapid clearance and destruction of naked plasmid in the body. The combination of Chi/DPO3-Dox and Chi/DPO7-pPEDF resulted in the strongest inhibition of tumor growth, bone lysis and metastasis to lungs. It was expected that this combination could provide a dual-pronged regime for OS treatment.

Acknowledgement

This project was supported by the University of Melbourne and St Vincent's Hospital (Australia). We would like to thank Mr. Bruce Abaloz (Department of Zoology, the University of Melbourne) for his help with the histology analysis.

Appendix

Figures with essential colour discrimination. Figs. 3, 5 and 6 in this article are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.05.035.

References

[1] Longhi A, Errani C, De Paolis M, Mercuri M, Bacci G. Primary bone osteosarcoma in the pediatric age: state of the art. Cancer Treat Rev 2006;32:423–36.
[2] Quan GMY, Ojaimi J, Nadesapillai WAP, Zhou H, Choong PFM. Resistance of epiphyseal cartilage to invasion by osteosarcoma of antiangiogenic factors. Pathobiology 2002;70:361–7.
[3] Unni KK. Dahlin's bone tumors: general aspects and data on 11,087 cases. Philadelphia: Lippincott-Raven Publishers; 1996.
[4] Dass CR, Ek ET, Contreras KG, Choong PF. A novel orthotopic murine model provides insights into cellular and molecular characteristics contributing to human osteosarcoma. Clin Exp Metastasis 2006;23:367–80.
[5] Ferguson WS, Goorin AM. Current treatment of osteosarcoma. Cancer Invest 2001;19:292–315.

[6] Patel SJ, Lynch Jr JW, Johnson T, Carroll RR, Schumacher CRN, Spanier S, et al. Dose-intense ifosfamide/doxorubicin/cisplatin based chemotherapy for osteosarcoma in adults. Am J Clin Oncol 2002;25:489–95.
[7] Ek ET, Dass CR, Contreras KG, Choong PFM. Pigment epithelium-derived factor overexpression inhibits orthotopic osteosarcoma growth, angiogenesis and metastasis. Cancer Gene Ther 2007;14:616–26.
[8] Patil SD, Rhodes DG, Burgess DJ. DNA-based therapeutics and DNA delivery systems: a comprehensive review. AAPS J 2005;7:E61–77.
[9] Witlox MA, Lamfers ML, Wuisman PI, Curiel DT, Siegal GP. Evolving gene therapy approaches for osteosarcoma using viral vectors: review. Bone 2007;40:797–812.
[10] Dass CR, Contreras KG, Dunstan DE, Choong PFM. Chitosan microparticles encapsulating PEDF plasmid demonstrate efficacy in an orthotopic metastatic model of osteosarcoma. Biomaterials 2007;28:3026–33.
[11] Ta HT, Han H, Larson I, Dass CR, Dunstan DE. Chitosan–dibasic orthophosphate hydrogel: a potential drug delivery system. Int J Pharm 2009;371:134–41.
[12] Ta HT, Dass CR, Larson I, Choong PFM, Dunstan DE. A chitosan–dipotassium orthophosphate hydrogel for the delivery of doxorubicin in the treatment of osteosarcoma. Biomaterials 2009;30:3605–13.
[13] Fisher JL, Mackie PS, Howard ML, Zhou H, Choong PFM. The expression of the urokinase plasminogen activator system in metastatic murine osteosarcoma: an *in vivo* mouse model. Clin Cancer Res 2001;7:1654–60.
[14] Winter HH, Chambon F. Analysis of linear viscoelasticity of crosslinking polymer at gel point. J Rheol 1986;30:367–82.
[15] Chenite A, Gori S, Chive M, Desrosiers E, Buschmann MD. Monolithic gelation of chitosan solutions via enzymatic hydrolysis of urea. Carbohydr Polym 2006;64:419–24.
[16] Cho J, Heuzey M-C, Begin A, Carreau PJ. Effect of urea on solution behaviour and heat-induced gelation of chitosan-b-glycerophosphate. Carbohydr Polym 2006;63:507–18.
[17] Berlin O, Samid D, Donthineni-Rao R, Akeson W, Amiel D, Woods VL. Development of a novel spontaneous metastasis model of human osteosarcoma transplanted orthotopically into bone of athymic mice. Cancer Res 1993;53:4890–5.
[18] Becerra SP, Sagasti A, Spinella P, Notario V. Pigment epithelium-derived factor behaves like a noninhibitor serpin. Neurotrophic activity does not require the serpin reactive loop. J Biol Chem 1995;270:25992–9.
[19] Dawson DW, Volpert OV, Gillis P, Crawford SE, Xu H, Benedict W. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 1999;285:245–8.
[20] Volpert OV, Zaichuk T, Zhou W, Reiher F, Ferguson TA, Stuart PM. Inducer-stimulated Fas targets activates endothelium for destruction by anti-angiogenic thrombospondin-1 and pigment epithelium-derived factor. Nat Med 2002;8:349–57.
[21] Cai J, Jiang WG, Grant MB, Boulton M. Pigment epithelium-derived factor inhibits angiogenesis via regulated intracellular proteolysis of VEGFR-1. J Biol Chem 2006;281:3604–13.
[22] Pignolo RJ, Francis MK, Rotenberg MO, Cristofalo VJ. Putative role for EPC-1/PEDF in the growth arrest of human diploid fibroblasts. J Cell Physiol 2003;195:12–20.
[23] Abe R, Shimizu T, Yamagishi S, Shibaki A, Amano S, Inagaki Y. Overexpression of pigment epithelium-derived factor decreases angiogenesis and inhibits the growth of human malignant melanoma cells in vivo. Am J Pathol 2004;164:1225–32.
[24] Yoo S-H, Lee J-S, Park SY, Kim Y-S, Chang P-S, Lee HG. Effects of selective oxidation of chitosan on physical and biological properties. Int J Biol Macromol 2005;35:27–31.
[25] Varum KM, Myhr MM, Hjerde RJN, Smidsrod O. In vitro degradation rates of partially *N*-acetylated chitosans in human serum. Carbohydr Res 1997;299:99–101.
[26] Pangburn SH, Trescony PV, Heller J. Lysozyme degradation of partially deacetylated chitin, its film and hydrogels. Biomaterials 1982;3:105–8.
[27] Nordtveit RJ, Varum KM, Smidsrod O. Degradation of fully water-soluble, partially *N*-acetylated chitosans with lysozyme. Carbohydr Polym 1994;29:163–7.
[28] Peters CW, Kruse U, Pollwein R, Grzeschik KH, Sippel AE. The human lysozyme gene. Sequence and chromosomal localization. Eur J Biochem 1989;182:507–16.
[29] Jolles J, Jolles P. Human tear and human milk lysozyme. Biochemistry 1967;6:411–7.
[30] Hankiewicz J, Swierczek E. Lysozyme in human body fluids. Clin Chim Acta 1974;57:205–9.
[31] Venezie RD, Towes AD, Morell P. Macrophages recruitment in different models of nerve injury: lysozyme as a marker for active phagocytosis. J Neurosci Res 1995;40:99–107.
[32] Montagne P, Cuiliere ML, Mole C, Bene MC, Faure G. Microparticle-enhanced nephelometric immunoassay of lysozyme in milk and other human body fluids. Clin Chem 1998;44:1610–5.
[33] Sambrook J, Russel DW. Molecular cloning: a laboratory manual, 3rd ed. New York: Cold Spring Harbor Laboratory Press; 2001.
[34] Sigma–Aldrich. Biochemicals, reagents & kits for life science research. Sigma–Aldrich; 2006.
[35] Zhang J, Chua LS, Lynn DM. Multilayered thin films that sustain the release of functional DNA under physiological conditions. Langmuir 2004;20:8015–21.
[36] Shea LD, Smiley E, Bonadio J, Mooney DJ. DNA delivery from polymer matrices for tissue engineering. Nat Biotechnol 1999;17:551–4.
[37] Klugherz BD, Jones PL, Cui X, Chen W, Meneveau NF, DeFelice S, et al. Gene delivery from a DNA controlled-release stent in porcine coronary arteries. Nat Biotechnol 2000;18:1181–4.
[38] Luo D, Saltzman WM. Synthetic DNA delivery systems. Nat Biotechnol 2000;18:33–7.