Cell viabilities and biodegradation rates of DNA/protamine complexes with two different molecular weights of DNA

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Abstract

Two types of DNA/protamine complexes were prepared from protamine sulfate and 7000-bp DNA or original DNA to investigate the effect of the molecular weight of DNA on zeta potential, cell viability, flowability, soft tissue response and biodegradation rate. The 7000-bp DNA/protamine complex had a negative charge while the original DNA/protamine complex had a positive charge. Cell viabilities (90.4 %~106.8%) of the complexes were close to each other. The 7000-bp DNA/protamine complex became softer dough by kneading with water than original DNA/complex. *In vivo*, the original DNA/protamine complex showed a milder tissue response. The original DNA/protamine complex almost disappeared 30 days after implantation. The 7000-bp DNA/complex disappeared approximately 2 weeks after implantation and the areas where samples were implanted became empty. Thereafter, the empty space was gradually replaced by new soft tissues. The original DNA/protamine complex showed low intercalation and groove-binding ratios of DH. Results indicate that high DNA condensation by cationic protamine protected penetration of degradation enzymes into these complexes. It was found that high molecular weight of DNA reduced the biodegradation rate and flowability. The present study suggests that DNA/protamine complexes could be candidates for biomaterials that control the biodegradation rate and flowability.

Key words: DNA; protamine sulfate; DNA/protamine complex; DNA molecular weight; biodegradation rate

INTRODUCTION

Deoxyribonucleic acids (DNAs) are one of the candidates for biomaterials because they have many favorable characteristic properties for biomaterials. For example, they can intercalate and bind antibiotics or proteins between stacked base pairs or in grooves[1-3], and are less antigenic than other macromolecules such as proteins[4]. Furthermore, DNA may be a good material for making a bone-guiding scaffold because it has many phosphate groups that have a strong affinity for calcium. However, DNAs alone cannot be used as biomaterials because they are water-soluble and difficult to mold. These problems need to be solved in order to apply DNA as a biomaterial.

For the application of DNA as a biomaterial, we have tried to prepare various kinds of water-insoluble molecule DNA/polycation molecule complexes[5-9] and evaluated their mold fabrication[7], flowability[7,9] , cell viability[5,6,8], and biodegradability[5-9]. For example, Fukushima *et al.*[5-9] prepared water-insoluble DNA/polycation molecule complexes by reacting DNA with polycation materials, such as chitosan, cationic poly-amino acids, and protamine, in order to convert water-soluble DNA into water-insoluble DNA and improve the formability of DNA. Of these complexes, Fukushima *et al.*[9] reported that a water-insoluble 300-bp DNA /protamine complex powder became paste by kneading it with water and that the paste had suitable viscosity for clinical use. They also demonstrated that the paste caused no damage to osteoblast cells, mild soft tissue responses, or antibacterial effects against gram-positive bacteria. Moreover, the paste had good biodegradability and disappeared 10 days after implantation in soft tissue. The biodegradation speed of the paste also seemed to be faster than that of commercial biodegradable polymers such as collagen and polylactic acid [10].

Nair and Laurencin[11] described that natural polymers such as polysaccharides and proteins seem to be the obvious choice for biomedical applications due to excellent biocompatibility and are biodegradable by an enzymatic or hydrolytic mechanism. They are being investigation as suitable scaffold matrices mainly for low load-bearing applications in tissues engineering because of low mechanical properties or as matrices for controlled drug delivery. In addition to theses, we have expected that DNA/protamine complexes have potentials of being matrices for bone and soft tissues engineering or for controlled drug delivery because they consisted of natural polymers.

However, natural polymers have a disadvantage because fine-controlling of the degradation rate may be less well than that of synthetic biodegradable polymers. Aurer and Jorgić-Srdjak[10] demonstrated in their periodontal membrane study that it was not possible to control the disintegration of resorbable membranes because of their inherent nature, and the disintegration speed can vary considerably among individuals, particularly for materials requiring enzymatic degradation such as collagen. Lu *et al.*[12] reported that degradation behavior is important for tissue-engineered cell/polymer construction involving cell growth and tissue regeneration for long-term performance. Nair and Laurencin[11] described that the rate of degradation of materials should match the intended application and should not be higher than a new matrix synthesis *in vivo*. Similarly in drug delivery systems, fine-tuning of drug release kinetics is possible by varying the degradation rate of the matrix polymer.

We consider that the degradable speed of biodegradable biomaterials such as DNA/cationic polymer complexes has a critical impact on tissue engineering and drug delivery systems, and that biomaterials may be capable of wide clinical applications if their degradable speed could be adjusted.

It is well known that the degradation rate of polymer can be controlled by adjusting molecular weight[12] and porosity[13], and cross-linking[14]. First, we have made an attempt to control the degradation rate of the complexes by the use of DNA with different molecular weight.

In a previous study[15], an original DNA (bp > 20,000)/protamine complex was prepared to investigate the degree to which the degradation rate of a DNA/protamine complex could be delayed using original DNA with a super high molecular weight. It was found that original DNA/protamine complex powder was biodegraded with mild soft tissue responses and almost disappeared 30 days after implantation in rat soft tissues. Cell viability seemed to be slightly lower than that of the 300-bp DNA/protamine complex reported by Fukushima *et al.*[8].

However, the effect of different molecular weights of DNA on the properties of a DNA/protamine complex, including degradation rate, is still not clear. In particular, an investigation of a DNA/protamine complex with a middle range molecule weight, which may be thousands of base pairs, is needed to more clearly understand the properties of DNA/protamine complexes. Concurrently, it is noted that polymer molecular weight can influence biological activity *in vivo*[16].

The purpose of this study is to evaluate the effect of difference molecular weight on cell viability *in vitro* and in the DNA/protamine complexes on the biodegradation behavior *in vivo* and histopathological responses using DNA/protamine complexes with 7000-bp DNA and original DNA.

Secondly, we evaluate the role of difference molecular weight of DNA related to molar binding ratio of DNA to protamine, preparation yield, Zeta potential and porosity of the complexes, and consistency of the complexes with distilled water.

MATERIALS AND METHODS

Preparation of DNA/protamine complexes

The synthesis scheme for the DNA/protamine complexes is shown in Fig.1. Sterilized salmon testis DNA (7000-bp DNA, Maruha-Nichiro Holdings, Ltd., Tokyo, Japan) cleaved with nuclease into 7000-bp fragments, sterilized salmon testis DNA (more than 20,000bp DNA, Maruha-Nichiro Holdings, Ltd., Tokyo, Japan) obtained by the ethanol precipitation method, and 2% sterilized salmon testis protamine sulfate (mol. wt. 4500) solution (Maruha-Nichiro Holdings, Ltd., Tokyo, Japan) were used in the present study.

The 7000-bp DNA (500 mg) and original DNA (500 mg) were dissolved in 200 mL distilled water. Protamine sulfate solutions at concentrations of 0.25% were prepared by adding distilled water to 2% sterilized salmon testis protamine sulfate solution. DNAs in 200 mL distilled water were added to protamine sulfate solution (200 mL). The mixtures were stirred at 20°C for 1 h. DNA/protamine complex powder was washed with distilled water for 24 h after decantation. DNA/protamine complexes were then collected by centrifugation at 9000 rpm for 10 min and washed with distilled water. This process was repeated twice. Complexes were cracked with a frozen cell crasher (CRYO-PRESS, Microtec Nition, Funabashi, Japan) after they were frozen in liquid nitrogen and then dried for 24 h in a FD-5N freeze-dryer (Eyela, Tokyo, Japan). Each freeze-dried complex powder was sieved to obtain powder with grain sizes between 200 μm and 300 μm for all experiments, except for the cell viability assay and zeta potential measurements. All procedures were carried out under sterile conditions and with sterilized instruments and materials.

Determination of the binding ratios of DNA to protamine in DNA/protamine complexes

The binding ratios of DNA to protamine in DNA/protamine complexes were estimated from the amount of incorporated phosphate. Phosphate was determined using the molybdenum blue method[17] and measurement with a U/2001 spectrophotometer (Hitachi Ltd., Tokyo, Japan). Measurements were performed in triplicate. The DNA-protamine binding ratio was calculated according to a previous study[5].

Zeta potential measurements of DNA /protamine complexes

An electrophoretic light-scattering spectrophotometer (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, Worcestershire, UK) was used to measure the zeta potential of the complexes. Each freeze-dried complex powder was sieved to obtain powder with a grain size of more or less 45 μm. Particles suspended in 10 mM sodium chloride solution (pH 7.4) were electrophoretically moved in sample cells to measure the zeta potential.

Field emission-scanning electron microscopic (FE-SEM) observation of porosity in DNA/protamine complexes

DNA/protamine complexes were freeze-fractured to observe their interior structures. The fractured surface was coated with evaporated carbon and was then observed with a JSM-6330F field emission-scanning electron microscope (JEOL, Tokyo, Japan)

Cell viability assay

Each freeze-dried complex powder was sieved to obtain powder with grain sizes between 45 μm and 100 μm. Complex powder was sterilized by irradiation with an electron beam accelerator (Dynamitron, IBA Industrial, Inc., Sterling, VA, USA) for 1.9 sec. at 20 KGy.

MC-3T3-E1 mouse osteoblast-like cells (Riken BioResource Center, Riken Tsukuba Institute, Tsukuba, Ibaragi, Japan) were cultured in α minimum essential medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA).

A 2-mL suspension of cells (1×10^4 cells/mL) was added into each well of a 6 well multiplate and incubated for 1 day at 37°C in a humidified 5% CO_2 atmosphere. After the 1-day incubation, the medium was removed and cells were washed with Dulbecco's phosphate-buffered saline (Invitrogen Corp., Carlsbad, CA, USA).

A 2-mL suspension medium of pulverized complexes (0.5 mg, 1 mg, and 2 mg) was poured into each well and incubated for 5 days at 37°C in a humidified 5% CO_2 atmosphere. After the 5-day incubation, the medium was removed and cells were washed with Dulbecco's phosphate-buffered saline. Two mL of medium containing 0.2 mL of Cell Counting Kit-8 Aqueous (Dojindo Laboratories, Masuki, Kumamoto, Japan) was added to each well, and the plate was incubated for 2 h at 7°C in a humidified atmosphere containing 5% CO_2. Optical density was measured using a plate reader (Multiskan JX, Labsystems Oy, Helsinki, Finland) at 450 nm as the detection wavelength and at 650 nm as the reference wavelength. Cell viability was expressed as a percentage compared to untreated cells. All experiments were performed in quintuplicate. Cell viability was analyzed statistically using a two-way analysis of variance (ANOVA) and Fisher projected least significant difference (Fisher's PLSD)

multiple comparison tests to determine significant differences in cell viability among samples at the 5% level of significance.

Consistency measurement of DNA/protamine complex paste

Complex powder (1.5 g) and 1.2 mL of distilled water was kneaded in a mortar with a pestle and then extra water was absorbed with paper. Kneading followed by absorbing was repeated two more times. The pastes were filled into a silicone mold (5-mm internal diameter and 0.5-mm height) on a Teflon plate. The top surface of the paste was covered by a Teflon plate to flat and then the covered Teflon plate was removed. After carefully removing bottom Teflon plate and silicone mold, the paste disks were put on the glass plate and then another glass plate was carefully put on surface of the paste disks. The assembly was placed on surface of metal disk in a constant load compression testing apparatus for multiple purposes (A001, JAPAN MECC CO., LTD., Tokyo, Japan) for loading. The paste disks were loaded continuously at 1 kg or 6 kg and 20°C for 90 sec. The diameters of one paste disk at three locations were measured with caliper after loading and the mean diameter was calculated as consistency for each sample. Each consistency was obtained from three samples. The consistency (diameter) of the paste disks was analyzed statistically using two-way analysis of variance (ANOVA) and Fisher's PLSD multiple comparison tests to determine statistical differences in cell viability among the samples at the 5% level of significance.

Disk preparation of DNA /protamine complex powder

A complex disk was prepared for animal experiments. DNA/protamine complex powder (100 mg) and 0.1 mL of distilled water were mixed in a mortar with a

Teflon spatula. The mixture of powder and water was put into a silicone mold (5 mm internal diameter and 1.5 mm height) with the end covered by a Teflon plate and then the top surface of the complex was covered by a Teflon plate. Fabricated complex disks (40 mg) were immediately and carefully removed from the Teflon plate and silicone mold.

All procedures were carried out under sterile conditions and with sterilized instruments and materials on a clean bench.

Implantation and histological evaluation

Animal experiments (No 10026) were performed in accordance with the Ethical Guidelines for Animal Experiments of Fukuoka Dental College. All disks were sterilized by irradiation with an electron beam accelerator (Dynamitron, IBA Industrial Inc., Sterling, VA, USA) for 1.9 sec. at 10 kGy.

Experiments were conducted using 10-week-old male Sprague-Dawley rats weighing approximately 200 g. Surgery was performed under general anesthesia induced by 2% isoflurane (Abbott Laboratories, Abbott Park, IL, USA) and an air mixture gas with a flow rate of 1.0 L/min using an anesthesia gas machine (Anesthesia machine SF-B01, MR Technology Inc., Tsukuba, Ibaraki, Japan). An incision was made in back of the rats and fabricated complex disks were implanted subcutaneously. A surface of skin in the implant site was marked by a thread. After insertion of the samples, soft tissues were closed in separate layers by suturing with intracutaneously resorbable Vicryl 3-0 (Ethicon Inc., Somerville, NJ, USA). Rats (24 in total) were divided into four groups for different observation times (3, 7, 21, and 30 days). Each rat received one sample disk. Three rats were used for each group at each time point. At

each time point, animals were sacrificed with an overdose of isoflurane.

After predetermined intervals of time, implanted materials were removed from subcutaneous tissue together with the surrounding tissue. Implants were fixed in 4% (w/v) paraformaldehyde in phosphate buffer (pH 7.4) and embedded in paraffin by routine procedures. Tissue sections, cut serially at 3 μm, and all serial sections were stained with hematoxylin and eosin (HE) for histological observation. The observation was used by a Nikon Eclipse 55i light microscope (Nikon, Tokyo, Japan). Histological points of view contained remain of implant materials, foreign body tissue reaction, and tissue repair. Histological grading was evaluated by absorption of materials and tissue reaction for repair process in four time points.

Determination of the binding amounts of daunorubicin hydrochloride to complexes

Each DNA/protamine complex powder (50 mg) was immersed for 48 h at 20°C in 5 mL of daunorubicin hydrochlorid**e** (DH) solution (1.8×10^{-3}mole/mL). The absorbance from 400 nm to 600 nm of each solution was measured before and after the reaction using a V-560 spectrophotometer (Jasco Corp., Tokyo, Japan). The amounts of intercalated and bound DH in the complexes were calculated from the difference in the total peak area for absorbance from 400 to 580 nm before and after the reaction. All experiments were performed in triplicate.

RESULTS

Preparation of DNA/protamine complexes

White water-insoluble DNA/protamine complexes were obtained from the reaction of DNA with two different molecular weights and protamine. For both complexes, the molar binding ratio of one base-pair of DNA to protamine, which was calculated from analysis of the phosphate content, was 0.1 (Table 1).

The yields of complex formation were close to each other, where 7000-bp DNA/protamine and original DNA/protamine complex yields were 69.3% and 67.7 %, respectively (Table 1).

Z (zeta) potentials of complexes

Z potentials of each complex are shown in Table 1. The DNA/protamine complex with 7000-bp and the original DNA/protamine complex showed Z potentials of -33.3 mV and 24.3 mV, respectively, at pH 7.4. These Z potential values demonstrated that the 7000-bp DNA/protamine complex has a negative charge and the original DNA/protamine complex has a positive charge.

Observation of the internal structure of DNA/protamine complexes by FE-SEM

Figure 2 shows FE-SEM of the fractured surface of the complex. Both complexes had a porous structure. The areas with pores in the 7000-bp DNA/protamine complex were larger than those in the original DNA/protamine complex.

Cell viability assay

Cell viabilities of DNA/protamine complexes are shown in Table 2. A two-way ANOVA; Statview J 5.0 followed by Fisher's PLSD multiple comparison tests showed

no significant differences in cell viability between the types of sample (p>0.05), and significant differences in cell viability between the amounts of sample (p<0.05). A two-way interaction was not found for the sample amount or type (p > 0.05). There were also no significant differences in cell viability between different samples at 0.5 mg/mL, 1.0 mg/mL, and 2.0 mg/mL (p > 0.05), but cells treated with 1 mg/mL and 2 mg/mL of the 7000-bp DNA/protamine complex showed a significantly lower viability than cells treated with 0.5 mg/mL (p < 0.05).

Consistency measurement of the DNA/ protamine complex paste

Both complexes became paste-like materials after mixing with water. Paste-like materials of the 7000-bp DNA/protamine complex and original DNA/protamine complex were like dough and hard dough, respectively. Both dough-like complexes could be filled into the mold with a spatula, but could not be injected with a syringe.

Consistencies of the complex dough were shown in Fig. 3. A two-way ANOVA; Statview J 5.0 followed by Fisher's PLSD multiple comparison tests showed significant differences in consistency between the types of sample (p<0.05), and significant differences in consistency between the weight of load (p<0.05). A two-way interaction was not found for the sample amount or type (p > 0.05). The consistencies of 7000-bp DNA/protamine complex dough were significant higher than those of original DNA/protamine complex dough at 1 kg and 6 kg of loads ((p<0.05).

Implantation and histological evaluation

To compare the biocompatibility and biodegradability of both complexes, we examined histological changes in implant materials in the subcutaneous tissues of rats

(Figs 4 and 5).

Biodegradation started in the 7000-bp material on day 3 after implantation, showing an empty space in the center of the implant site (Fig. 4 a). The remainder of the material was attached to the periphery of the implant site, which were circumscribed by cellular granulation tissue (Fig. 4d). Implant sites of original complexes showed capsulation of whole material by cellular granulation tissue (Figs. 5 a and b). The remaining materials appeared histologically to be similar to the same volume of materials at the time of implantation. By day 7 after implantation, the 7000-bp material was totally absorbed and the implant site showed a dilated, empty space surrounded by granulation tissue (Figs. 4 b and e). In implant sites of the original complex, biodegradation of materials started on day 7, showing fragmentation of materials (Figs. 5 b and e). After day 21, the empty space of the 7000-bp implant was totally replaced by dense fibrous connective tissue, similar to the surrounding subcutaneous tissue (Figs. 4 c and f). After day 30, histological evaluation was almost the same as that on day 21 for the 7000-bp DNA/protamine complex.

The progress of biodegradation of materials was observed in the implant site of the original DNA/protamine complex on day 21 (Figs. 5 c and f). The implant site was replaced by fibrous granulation tissue containing small fragments of materials. On day 30, replacement of fibrous granulation tissue progressed in the site of the original complex implant, but a few materials still remained (Figs. 5 d and g).

Determination of the binding of complexes to daunorubicin hydrochloride

Figure 6 shows intercalation and groove binding of DH within DNA/protamine

complexes. DH intercalated and bound in the grooves of DNA within complexes. After 48 h, the amount of intercalated and groove-bound DH in 7000-bp DNA/protamine and original DNA/protamine complexes were about 4.83 to 4.86 mg, and about 3.28 to 3.31 mg, respectively. These results suggested that one molecule of DH intercalated and groove-bound to approximately 5.2 bp of DNA in the 7000-bp DNA/protamine complex and to approximately 7.7 bp of DNA in the original DNA/protamine complex.

DISCUSSION

It is well known that α-helices of protamine lie in the major grooves of DNA, where they neutralize the negatively charged phosphate backbone[18], and one cycle of DNA contained approximately 10 base pairs[19]. We estimate that the obtained DNA/protamine complexes have one molecule of protamine per 10 base pairs of DNA and correspond to a 0.1 molar binding ratio of one base-pair in DNA to protamine. In this study, both complexes showed 0.10 molar binding ratios (Table 1). These values were very close to the theoretical value, but the original DNA/protamine complex showed a lower value than that (0.129) of a previous paper[15]. There are two more different results from this paper regarding the viability on the original DNA/protamine complex and preparation yield of the original DNA/protamine complex[15]. In the present study, cell viabilities of the original DNA/protamine complex were from 106.8 to 90.4% as shown in Table 2. Cell viabilities were slightly higher than those (99.0%~87.1%) in the previous paper[15]. The preparation yield (67.7%) of the original DNA/protamine complex was lower than that (75.0%) in the previous paper[15]. We consider that non-groove bound protamine to DNA may be more easily removed from DNA than

groove bound protamine. Fukushima *et al.*[8] reported that protamine alone seriously affected the cell viability of MC 3T3-E1. In the present study, washing of the complexes after preparation was longer than that in the previous study. We consider that long washing of the complex after preparation removed some of the non-groove bound protamine. As a result, it led to lower molar binding ratios of DNA to protamine in the original DNA/protamine complex, higher cell viabilities of the original DNA/protamine complex, and lower preparation yields.

In the present study, it was also found that the binding ratio of DNA to protamine, the preparing yield of the complex, and the cell viability with the complex were not dependent upon the molecular weight of DNA in the DNA/protamine complex. These results were very similar to those in the 300-bp DNA/protamine complex study reported by Fukushima *et al.*[8] .

In contrast, it seems that zeta potentials, consistency and biodegradation rates were dependent upon the molecular weights of DNA in DNA/protamine complexes. The 7000-bp DNA/protamine complex was softer after kneading with water and had a higher biodegradation rate than that of the original DNA/protamine complex. The surface of the 7000-bp DNA/protamine complex had a negative charge while that of the original DNA/protamine complex had a positive charge. Mady *et al.*[20] reported that the zeta potential of salmon sperm DNA was -21mV. Park *et al.*[21] reported that the zeta potential of plasmid DNA was -80mV. It seems that differences in molecular weights have an effect on the value of the zeta potential of DNA. We suspected that differences in the values of zeta potential between the 7000-bp DNA/protamine complex and original DNA/protamine complex were mainly dependent on the length of DNA in complexes. In generally, cationic polymers cause higher

cytotoxic effect than those with low cationic charge densities[22]. Thus, we predicted that DNA/protamine complex with higher zeta potential exhibits low cell viability. From the results in present study, for DNA/protamine complexes, the cell viability does not be influenced by the difference in zeta potential of the complexes.

Segura *et al.*[14] described that the clearance of biomaterials in vivo has limited their application in tissue engineering. We consider that strategies to reduce clearance in vivo involve fitting biomaterials in tissue defects for the development of new biomaterials. Fukushima *et al.*[9] reported that the 300-bp DNA/protamine complex became paste with 280.1 Pas of viscosity by kneading the complex powder and distilled water and this paste could be injected with a syringe, and described that paste is one favorable type of biomaterials because it can be easily formed into the desired shape and directly injected with a syringe into tissue defects with various shapes. In present study, we made an attempt to knead the complex powder with distilled water according in previous study[9]. We expected that the 7000-bp DNA/protamine complex became paste by kneading with distilled water because 7000-bp DNA was relatively low molecular weight, compared with original DNA. Contrary to our expectations, the 7000-bp DNA/protamine complex and original DNA/protamine complex became dough and hard dough that could not be passed through a commercial syringe with a needle of 0.25 mm internal diameter. However, for the 7000-bp DNA/protamine complex and original DNA/protamine complex, enlargement area ratios to their original surface areas were 2.0 and 4.2, or 1.8 and 3.4, respectively after measurement with loads of 1 kg and 6 kg (Fig. 3) and then their shape did not change under a condition of high humidity. The results indicated that their complex dough can be formed sheet with a hand and can be filled into tissue defects with simple shape using filling instruments.

Moreover, results suggested that if a DNA/protamine complex is required to be a paste, base pairs of the DNA have to be adjusted to less than 7000-bp. We expect that DNA/protamine complexes with more than 7000-bp DNA can be used for biomaterials by making shapes such as powder, blocks, and membranes.

As shown in Figs. 4 and 5, we demonstrated histologically that the 7000-bp DNA/protamine complex showed more susceptibility to biodegradation than the original DNA/protamine complex. In general, biodegradation processes of implant materials are divided into two steps, a humoral phase, which is an initial step of biodegradation that is mediated by extracellular enzymes from both blood plasma and cellular production, and a cellular phase, which is activated by phagocytes. In order to transport implant materials directly into phagocytes, extracellular enzymes must primarily degrade materials outside cells. In this study, differences in biodegradation speed between both complexes were responsible for the sensitiveness of extracellular enzymes. A satisfactory effect of degradation-related enzymes mainly depends on the penetrative activity of enzymes into implant materials because phagocytes almost were not observed beside both implanted complex. Our results for the original DNA/protamine complex imply that enzymes are too large to penetrate deeply into complexes and can act only on the complex surface. As a consequence, biodegradation of complexes remains a surface erosion process, with remainders of materials still existing 30 days after implantation. In contrast, the 7000-bp DNA/protamine complex was rapidly biodegraded and this biodegradation speed was very close to that of the 300-bp DNA/protamine complex reported by Fukushima *et al.* [9].

Yuan *et al.*[23] reported that DNA condensation by cationic polymers protected

DNA from enzyme degradation. As shown in Fig. 6, one molecule of DH bound to approximately 5.2 bp of DNA in the 7000-bp DNA/protamine complex, and 7.7 bp of DNA in the original DNA/protamine complex, respectively. Fukushima *et al.*[5] reported binding of one molecule of DH to approximately 4.5 to 6.2 bp of DNA in 300-bp DNA/chitosan complexes. These results suggest that the 7000-bp DNA/protamine complex may possess chemical characteristics to allow easy penetration of extracellular enzymes into the complex the same as the 300-bp DNA/protamine complex. The enzyme sensitivity of the 7000-bp DNA/protamine complex, which is superior to the original DNA/protamine complex, is supported by the findings of the internal structure of complexes by FE-SEM, which showed large areas with pores (Fig. 2). Furthermore, data of intercalation and groove-bound ratios of DH to complexes shown in Fig. 6 may also assist the susceptibility of the 7000-bp DNA/protamine complex to biodegradation. In implantation sites of the 7000-bp DNA/protamine complex, the degradation product was replaced by an empty space instead of granulation tissue. Fukushima *et al.*[8] reported that implantation sites of the 300-bp DNA/protamine complex, very similar to the biodegradation speed of the 7000-bp DNA/protamine complex, were replaced by granulation tissue during the degradation process. We speculate that the empty space is filled with an aqueous degradation product because of rapid biodegradation of the 7000-bp DNA/protamine complex by extracellular enzymes. The biodegradation mechanism of the DNA/protamine complexes from the obtained results is as follows: the complexes implanted in tissues are continuously biodegraded by enzymes such as nuclease Then, the biodegraded complexes begin to elute from the remained complexes because of water-solubility of biodegraded complexes. Finally, the complexes are completely disappeared by either continuous biodegradation or elution.

As described above, the DNA/protamine complexes could be intercalated and groove-bound by DH. It is well known that DNA can be intercalated and groove-bound by drugs such as netropsin, distamycin and adriamycin[1]. Although we did not investigate the potential of the DNA/protamine complexes for drug delivery systems in the present study, we expect that the DNA/protamine complex will be useful for drug delivery systems.

CONCLUSIONS

The 7000-bp DNA/protamine and original DNA/protamine complexes could be easily prepared from protamine sulfate and 7000-bp DNA or original DNA. The yields (approximately 70%) were very close to each other. The binding ratios of one base-pair of DNA to protamine in these complexes were almost equal. Cell viabilities (90.4 %~106.8%) of the complexes were very close. The 7000-bp DNA/protamine complex and original DNA/complex became dough and hard dough, respectively, by kneading with water and they could not be injected with a syringe. It was found that differences in the molecule weight of DNA can mainly influence flowability, zeta potentials, and biodegradation rates for DNA/protamine complexes. The 7000-bp DNA/protamine complex showed high flowability, high biodegradation rates, and a negative charge. The original DNA/protamine complex showed a milder soft tissue response and almost disappeared 30 days after implantation. It was found that the DNA/protamine complexes have little cytotoxicity and DNAs with super high molecular weights such as original DNA should be used if a DNA/protamine complex with a low biodegradation rate is required. The present study suggests that

DNA/protamine complexes are potential biomaterials for controlling biodegradation rate and flowability.

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Legends

Figure 1. Schematic illustration of DNA/protamine complexes

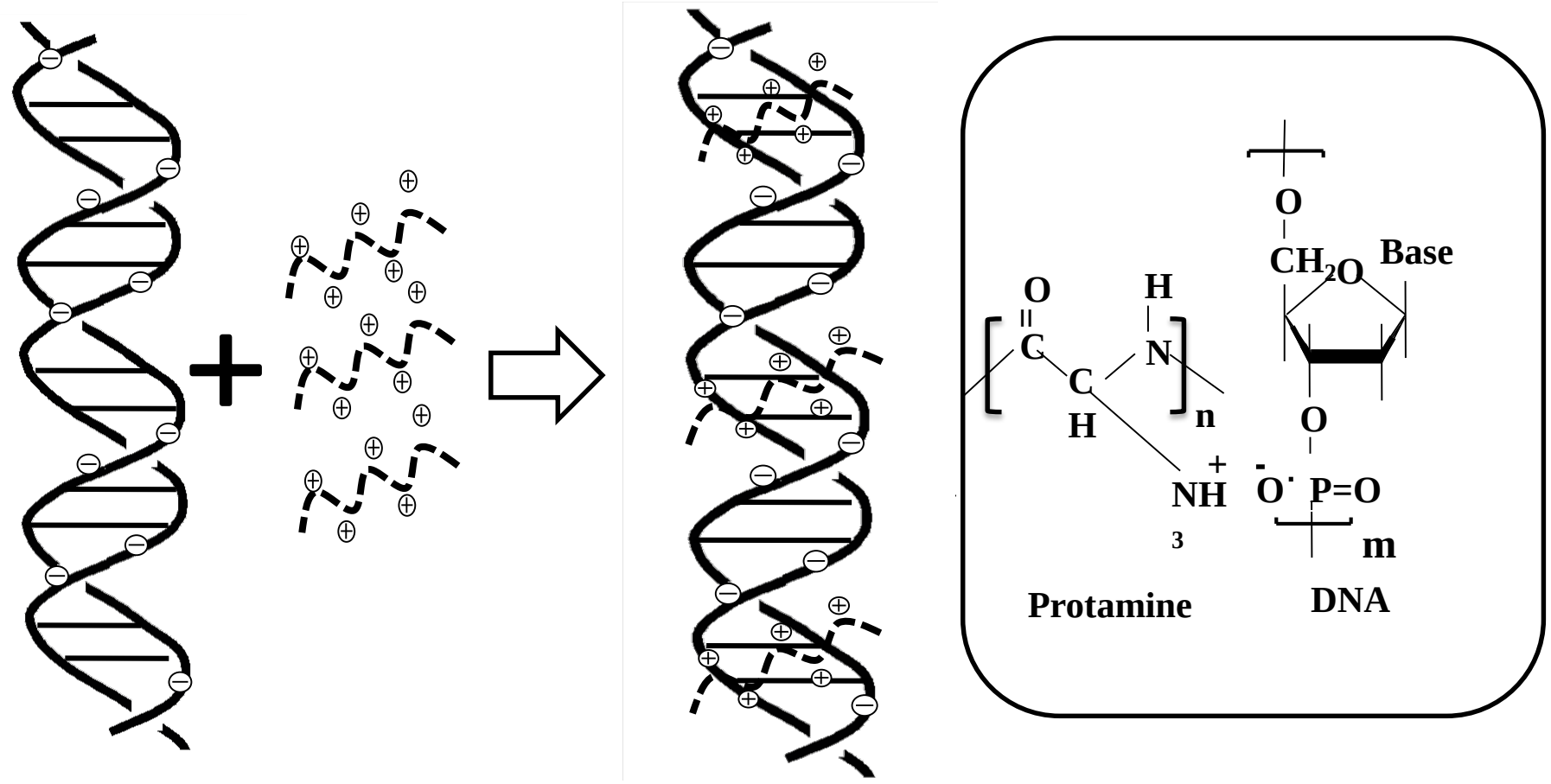
Figure 2. SEM photomicrograph of the fracture interior surface of the 7000-bp DNA/protamine complex (left) and original DNA/protamine complex (right) Bar=10μm

Figure 3. Consistency of the 7000-bp DNA/protamine complex (7000-bp) and original DNA/protamine complex (Original). Values with same letters show significant different ($p<0.05$).

Figure 4. Histopathology of subcutaneous tissue of skin implanted with 7000-bp DNA/protamine complex powder after 3 days (a), 7 days (b), and 21 days (c). (d)-(f) shows higher magnification of implant tissue on 3, 7, and 21 days, respectively. Arrow=sample Ellipse=sample implanted location Bars = 1 mm (a-c) and 200 μm (d-f).

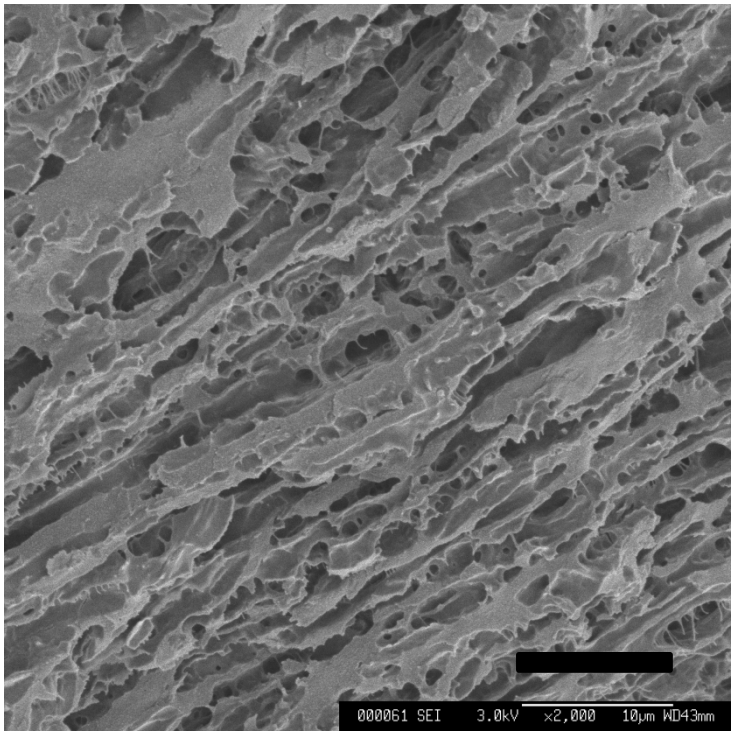
Figure 5. Histopathology of subcutaneous tissue of skin implanted with the original DNA /protamine complex after 3 days (a), 7 days (b), 21 days (c) and 30 days (d). (e)-(g) shows higher magnification of implant tissue on 3, 7, and 21 days, respectively. Bars = 1 mm (a-d) and 200 μm (e-g).

Figure 6. Intercalation and groove-binding of daunorubicin hydrochloride to the 7,000-bp DNA/protamine complex (7000-bp) and the original DNA/protamine complex(Original).



DNA Protamine sulfate DNA/protamine complex

FIG 1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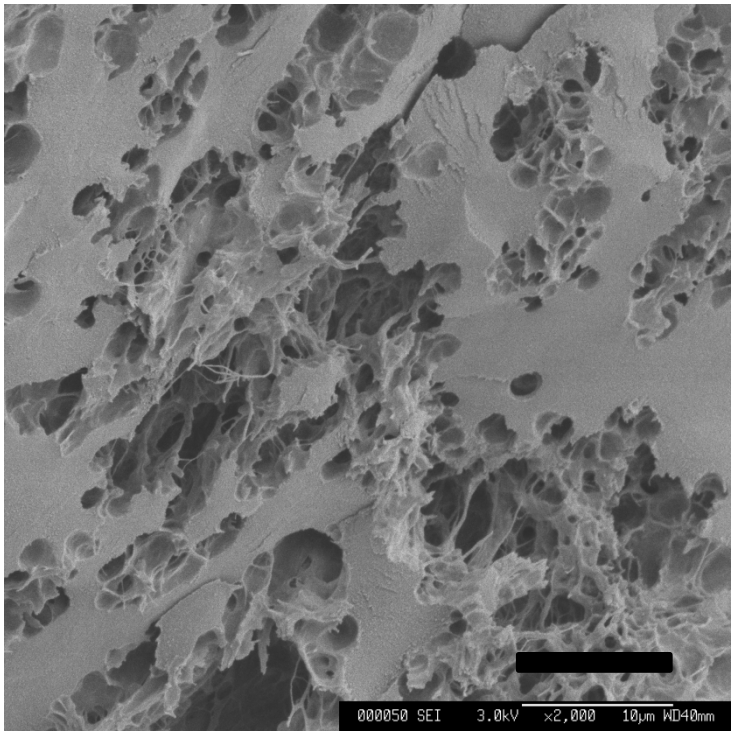
000050 SEI 3.0kV ×2,000 10μm WD40mm

FIG 2

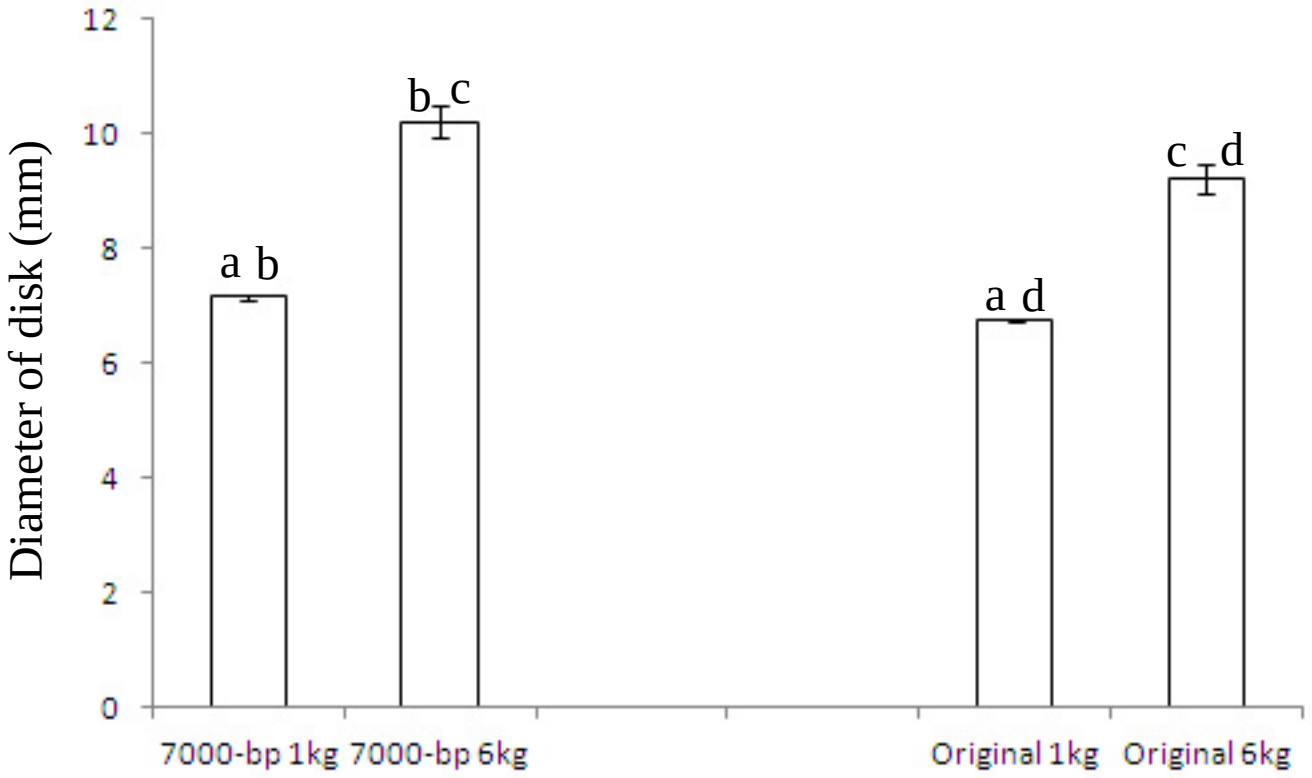
Diameter of disk (mm)
12
10
8
6
4
2
0
a b
b c
a d
c d
7000-bp 1kg
7000-bp 6kg
Original 1kg
Original 6kg

FIG 3

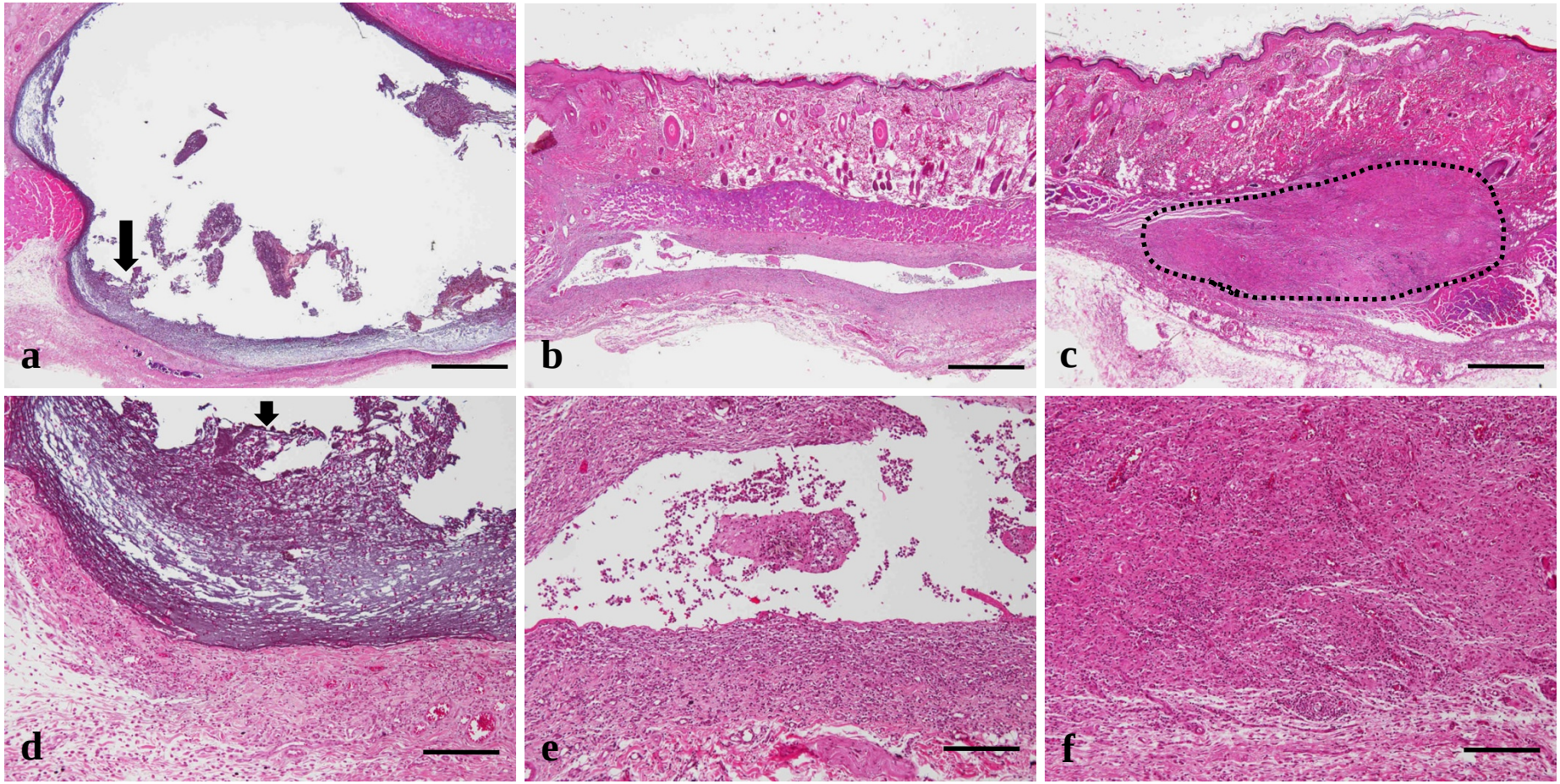

FIG 4

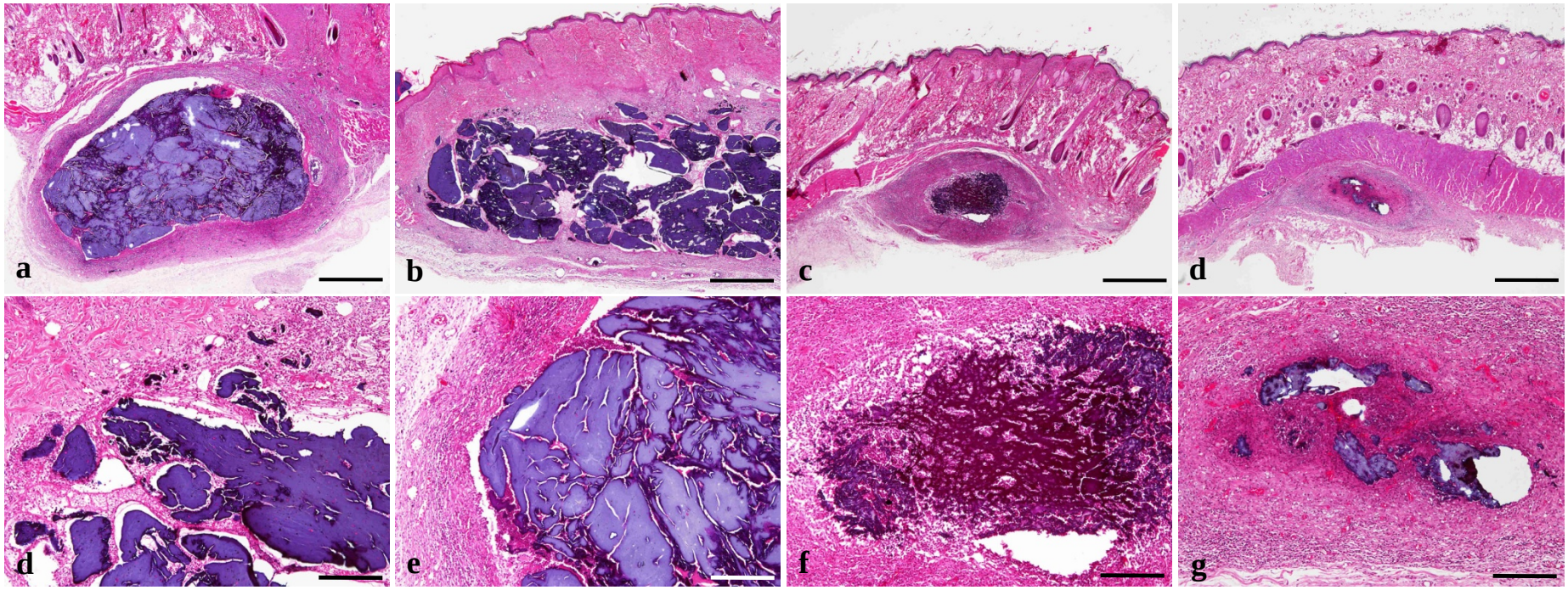

FIG 5

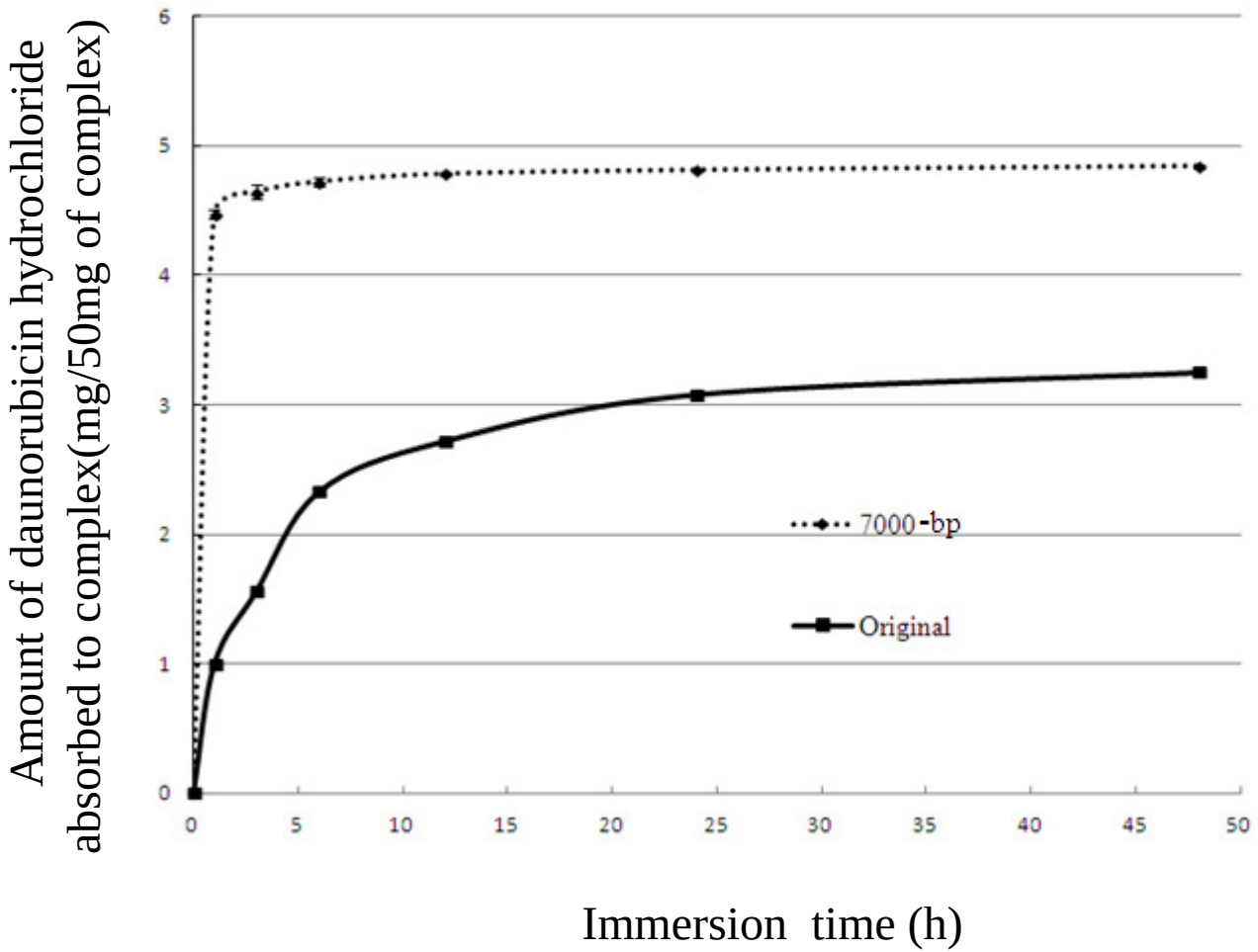
Amount of daunorubicin hydrochloride absorbed to complex(mg/50mg of complex)
0
1
2
3
4
5
6
0
5
10
15
20
25
30
35
40
45
50
7000-bp
Original
Immersion time (h)

FIG 6

Table 1 Amounts of prepared DNA/protamine complexes, molar binding ratios of DNA to protamine and zeta potentials of the complexes

Sample	One base-pair of DNA / molecule of protamine	Amount of prepared complex / Amount of starting material (%)	Zeta potential (mV)
7000-bp DNA/protamine complex	0.1	69.3	-33.3±1.5
Original DNA/protamine complex	0.1	67.7	24.3 ±4.0

Table 2 Effect of DNA/protamine complexes on Cell viabilities

Sample	Cell viability (% of control)		
	0.5mg/mL	1.0mg/mL	2.0mg/mL
7000-bp DNA/protamine complex	106.1± 5.5	104.9±5.1	95.7±1.9
Original DNA/protamine complex	106.8±12.0	100.5±5.6	90.4±7.7

Values connected by vertical and horizontal bars are not significant different ($p>0.05$).