J Mater Sci: Mater Med (2011) 22:2607–2615
DOI 10.1007/s10856-011-4446-9

DNA/protamine complex paste for an injectable dental material

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Received: 11 November 2010 / Accepted: 5 September 2011 / Published online: 16 September 2011

Abstract A DNA/protamine complex powder was prepared by reaction between DNA and protamine sulfate solution with stirring in order to develop a new injectable biomaterials for dental therapy. The powder of DNA/protamine complex became paste by kneading the complex powder and distilled water. Complex formation was confirmed by FT-IR measurement. The complex paste had a porous structure and its viscosity was approximately 280.1 Pas. The paste could easily pass through a needle of 0.25 mm internal diameter. It seemed that DNA/protamine complex paste has suitable viscosity for clinical use as an injectable biomaterial. Although, the complex paste delayed the growth speed of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Porphyromonas gingivalis* and *Prevotella intermedia* for limited periods, it cannot kill and inhibit growing bacteria. The complex paste disk showed a mild tissue response and gradually degraded after the implantation into the soft tissue of rats. These results suggested that this DNA/protamine complex paste could be a useful material for a biodegradable biomaterial. In particular, this paste will be applicable as an injectable biomaterial using syringe for the repair of defects of living tissue, GBR treatment and/or GTR treatment in dentistry.

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1 Introduction

Protamine is a series of arginine-rich polycationic proteins [1] that become highly α-helical on binding to DNA of spermatozoan nuclei of fish, birds and mammals among others [2, 3]. Some researchers have reported that protamine sulfate has antibacterial properties [1–3]. For example, Johansen et al. [2] reported that protamine from salmon killed growing gram-positive bacteria and significantly inhibited the growth of gram-negative bacteria in Tryptone Soy Broth. Protamine is also used as an antidote to heparin [2] and as a carrier of insulin [2], as well as a food additive for preventing the corruption of food [1, 3].

It is noted that DNA has some favorable characteristic properties for biomaterials. For example, antibiotics or proteins can be intercalated between stacked base pairs of DNA or bound in the grooves of DNA strands [4–6], and DNA is less antigenic than other macromolecules such as proteins or polysaccharides [7]. Fukushima et al. [8–11] prepared water-insoluble DNA/polycation complexes by the reaction of DNA with polycation materials such as

chitosan, poly-L-arginine, poly-L-histidine and poly-L-lysine to develop new types of biodegradable biomaterials. These DNA/polycation complexes showed very mild cell damage and mild soft tissue response [9, 10].

In a previous study [12], we prepared a DNA/protamine complex, which was porous water-insoluble white powder, by mixing an aqueous solution of DNA and that of protamine. It was found that this complex showed no damage to osteoblast-like cells and showed mild antibacterial activities in disk-diffusion test. Mild soft tissue response of the DNA/protamine complex was confirmed after implantation. It is suggested that DNA/protamine complex will have useful properties for dental materials.

Recent findings indicate that DNA/protamine complex powder becomes paste by kneading with water. It is expected that DNA/protamine complex paste can be easily injected into any shape of defect or cavity and then easily formed into the desired shape, such as a sheet. Moreover, it is easy for paste to be mixed with an inorganic or organic compound, such as hydroxyapatite or cytokines, to change the viscosity or facilitate tissue formation. In dentistry, injectable paste will be useful for guided tissue regeneration (GTR), for guided bone regeneration (GBR), as a drug carrier for gum pocket treatment, or for dental implant treatment.

In addition, one of the most important prerequisites for biomaterials is that they have adequate porosity for cell seeding, cell growth and extracellular matrix productions [13–15]. Fukushima et al. [11] in DNA/chitosan complex studies reported that reduction in porosity of DNA/chitosan complexes was allowed to significantly retard their biodegradation rate. Although, DNA/protamine complex paste has the advantages of handling as described above, it is considered that pasting takes the risk of deceasing porosity. Therefore, we investigated the characteristic of DNA/protamine complex paste.

In the present study, we prepared DNA/protamine complex paste and evaluated its viscosity, histopathological responses and antibacterial activities for the purpose of examining the possibility of using DNA/protamine complex paste as a dental material.

2 Materials and methods

2.1 Preparation of DNA/protamine complex

Sterilized salmon testis DNA provided by Maruha-Nichiro (Maruha-Nichiro Holdings, Ltd., Tokyo, Japan), which was cleaved with protease P3 into 300 bp fragments, and 2% sterilized salmon testis protamine sulfate (mol. wt. 4500) solution (Maruha-Nichiro Holdings, Ltd., Tokyo, Japan) were used in the present study.

DNA (500 mg) was dissolved in 100 ml of distilled water. The distilled water was added to 2% sterilized salmon testis protamine sulfate solution to prepare 0.5% protamine sulfate solution. DNA in 100 ml of distilled water was added to protamine sulfate solution (100 ml) and the mixture was stirred at 20°C for 1 h. The DNA/protamine complex was collected by centrifugation at 9000 rpm for 10 min and washed with distilled water. This process was repeated two times, after which the DNA/protamine complex was frozen in liquid nitrogen and then dried for 24 h in a FD-5 N freeze-dryer (Eyela, Tokyo, Japan). All procedures were carried out under sterile conditions and with sterilized instruments and materials.

2.2 Preparation of DNA/protamine complex paste

The freeze-dried DNA/protamine complex powder aggregation (Fig. 1A) was crushed in a mortar with a pestle to powder. The complex powder (1.5 g) and 1.2 ml of distilled water were kneaded in a mortar with a pestle and then any extra water was absorbed with paper. Kneading followed by absorbing was repeated two more times. The DNA/protamine complex paste (Fig. 1B) was filled into a syringe and then was injected from the syringe with a needle of 0.25 mm internal diameter (Fig. 1C).

For disk preparation of DNA/protamine complex paste, the DNA/protamine complex paste was injected into a silicone mold (5 mm internal diameter and 8 mm height) on a Teflon plate (Fig. 1D). The top surface of the complex was covered with a Teflon plate to flatten it and then the covered Teflon plate was removed (Fig. 1E). After carefully removing the bottom Teflon plate and the silicone mold, the complex paste disk was freeze-dried in accordance with the method for the preparation of DNA/protamine complexes (Fig. 1F).

All procedures were carried out under sterile conditions and with sterilized instruments and materials.

2.3 Infrared spectroscopy analyses of DNA, protamine sulfate, and DNA/protamine complex powder and paste

Infrared spectroscopy analyses were carried out with a fourier transform infrared (FT-IR) spectrometer (FT/IR-410, JASCO, Tokyo, Japan). For the analyses of original DNA, protamine sulfate and DNA/protamine complex powder, potassium bromide (KBr) pellets were prepared by grinding the samples with KBr and applying great pressure to the dry mixture. For the analyses of DNA/protamine paste, three films were prepared by pressing paste between Teflon plates. Each film was air-dried for 24 h or freeze-dried for 24 h after removing from Teflon plates.

Protamine sulfate and DNA/protamine complex powder were analyzed by transmission method. Original DNA and

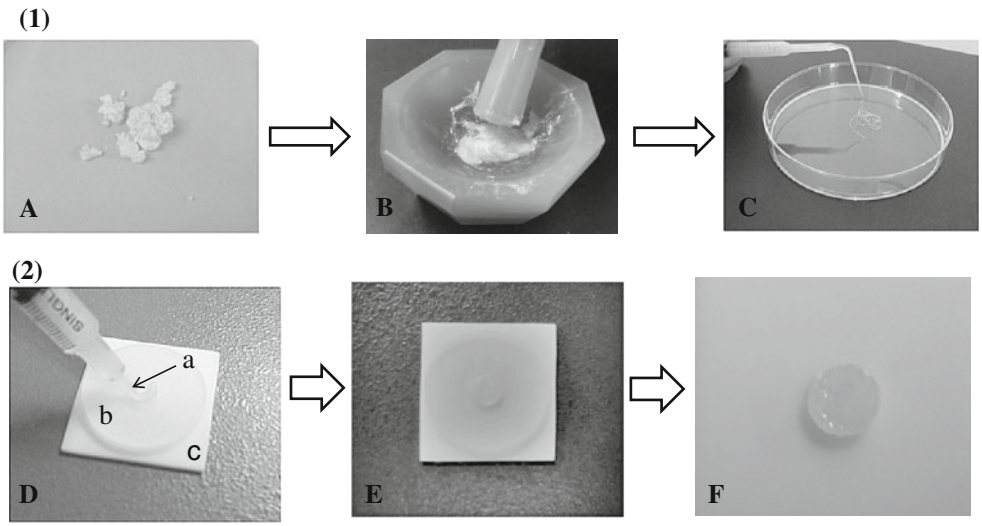

Fig. 1 Preparation paste **1** of DNA/protamine complex and preparation disk **2** of DNA/protamine complex paste. **A** DNA/Protamine complex powder. **B** DNA/Protamine complex paste prepared by mixing with water. **C** Injection of DNA/Protamine complex paste. **D** Injection of paste (*a*) into silicone mold (*b*) on Teflon plate (*c*). **E** Molded paste. **F** Fabricated disk

DNA/protamine films were analyzed by attenuated total reflectance (ATR) method.

2.4 Measurement of viscosity of DNA/protamine complex paste

The viscosity of DNA/protamine complex paste was measured with a capillary rheometer (Capillary Rheometer Shimadzu Flowmeter CFT-500D-PC, Shimadzu, Kyoto, Japan). A piston with 1 cm^2 cross section and a circular cross-sectional die of 1 mm in length and with a hole with a 1 mm diameter were used in this work. Tests were carried out at 28°C and with an extruding pressure of 0.49×10^6 Pa and repeated three times. The flow rates (Q) were determined on the basis of the following equation for the viscosity:

$$Q = A\frac{M}{10 \times \Delta t}(\mathrm{cm}^3/\mathrm{s})$$

where A is the cross section (cm^2) of the piston, M is the movement distance (mm) of the piston and Δt is the elapsed time (sec) for the movement of the piston. The shear rates (γ) were calculated on the basis of the following equation for the viscosity:

$$\gamma = \frac{32 \times Q}{\pi \times D^3} \times 10^3(\mathrm{s}^{-1})$$

where Q is the flow rate and D is the diameter (mm) of the hole in the die. The shear stress (τ) was calculated on the basis of the following equation for the viscosity:

$$\tau = \frac{P \times D}{4 \times L}(\mathrm{Pa})$$

where P is the extruding pressure (Pa) and L is the length (mm) of the hole in the die. The viscosities (η) were calculated on the basis of the following equation:

$$\eta = \frac{\tau}{\gamma}(\mathrm{Pas})$$

2.5 Field emission-scanning electron microscopic (FE-SEM) observation of porosity in DNA/protamine complex powder and DNA/protamine complex paste

DNA/protamine complex paste was freeze-dried in accordance with the method for the preparation of DNA/protamine complexes. DNA/protamine complex powder aggregation and DNA/protamine complex paste were freeze-fractured to observe their interior structures. The fractured surfaces were coated with evaporated carbon and were then observed with a JSM-6330F field emission-scanning electron microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 3.0 kV and a magnification of ×2,000.

2.6 Antibacterial activity test

Staphylococcus aureus 209P, *Pseudomonas aeruginosa* (laboratory stock), *Porphyromonas gingivalis* W83 and *Prevotella intermedia* ATCC25611 were used in this study. The growth media used were tryptic soy broth (TSB) and tryptic soy agar (TSA) (Becton, Dickinson and Co., USA) for *S. aureus* and *P. aeruginosa*, TSB supplemented with 5 μg/ml hemin and 1 μg/ml menadione and Brucella HK agar containing 5% rabbit blood (Kyokuto Pharmaceutical Ind., Tokyo, Japan) for *P. gingivalis*, and GAM broth (Nissui, Tokyo, Japan) and Brucella HK agar for *P. intermedia*.

As a preculture, a single colony of *S. aureus* or *P. aeruginosa* on each agar plate was inoculated in a broth (5 ml) at 37°C for 16 h with shaking. A single colony of *P. gingivalis* or *P. intermedia* on each agar plate was inoculated in a broth (5 ml) at 37°C for 24–48 h under anaerobic conditions (10% CO_2, 10% H_2, 80% N_2).

The precultures mentioned above diluted in each broth were inoculated into 5 ml of new broth. Three DNA/protamine complex disks were added into each inoculated broth. The growth of each bacterium was monitored with a Perkin-Elmer Junior model 35 spectrophotometer at an optical density of 625 nm (OD625).

Antibacterial activity was analyzed statistically with two-way analysis of variance (ANOVA) between the control (Teflon plate disk) and paste disks at a 5% level of significance.

2.7 Implantation and histological evaluation

Animal experiments were performed in accordance with the ethical guidelines for animal experiments of Fukuoka Dental College. Six-week-old male Sprague–Dawley rats weighing approximately 200 g were used in the present study. Surgery was performed under general anesthesia induced using 2% isoflurane (Abbott Laboratories, Abbott Park, IL, USA) and air mixture gas flow at 1.0 l/min using an anesthesia gas machine (Anesthesia machine SF-B01, MR Technology, Inc., Tsukuba, Ibaraki, Japan). An incision was made at the skin of rats and the DNA/protamine complex paste disk was implanted into the soft tissue of rats. After the insertion of samples, the soft tissues were closed in separate layers by suturing with intracutaneously resorbable Vicryl 3-0 (Ethicon, Inc., Somerville, NJ, USA). The control was a group of sham-operated rats that did not undergo sample implantation. The rats (24 in total) were divided into two groups (one experimental group and one control group) for different observation times (3, 10, 15 and 20 days). Three rats were used for each group at each period. At each period, the animals were sacrificed by injection with an overdose of isoflurane. After sacrificing the animals, the soft tissues containing the implanted samples were immediately excised. They were fixed in 4% (w/v) paraformaldehyde in phosphate buffer (pH 7.4), dehydrated with graded alcohols, cleared in xylene and embedded in paraffin by routine procedures. The specimens were sectioned at 3 μm. Three tissue sections were prepared for each group at each period. The sections were stained with hematoxylin and eosin for histological observation using a Nikon Eclipse 55i light microscope (Nikon, Tokyo, Japan) at a magnificent of ×100 and ×200.

3 Results

3.1 Preparation of DNA/protamine complex and DNA/protamine complex paste

Water-insoluble DNA/protamine complex white powder was obtained from the reaction of native DNA with protamine. DNA/protamine complex white powder was aggregated and then sedimented by centrifugation. The powder aggregation was finely crushed in a mortar with a pestle. The complex paste was prepared by kneading the complex powder and distilled water. The paste could pass through a needle with 0.25-mm internal diameter (Fig. 1C).

3.2 Preparation of DNA/protamine complex paste disk

Figure 1D–F shows the procedure of DNA/protamine complex paste disk preparation. The paste could be easily injected into the mold and the paste disk could be easily prepared by removing the mold. The form of the disk was very stable under the humidity conditions used.

3.3 Infrared spectroscopy analyses of DNA, protamine sulfate, and DNA/protamine complex powder and paste

FT-IR spectra of DNA, protamine sulfate, and DNA/protamine complex powder are shown in Fig. 2. Figure 2A shows FT-IR spectrum of original DNA. The broad peak around 3200 cm^{-1} was attributed as OH of bound water. The peaks around 1600, 1200, and 1050 cm^{-1} could be assigned as C=O, P=O and P–O–C stretching modes,

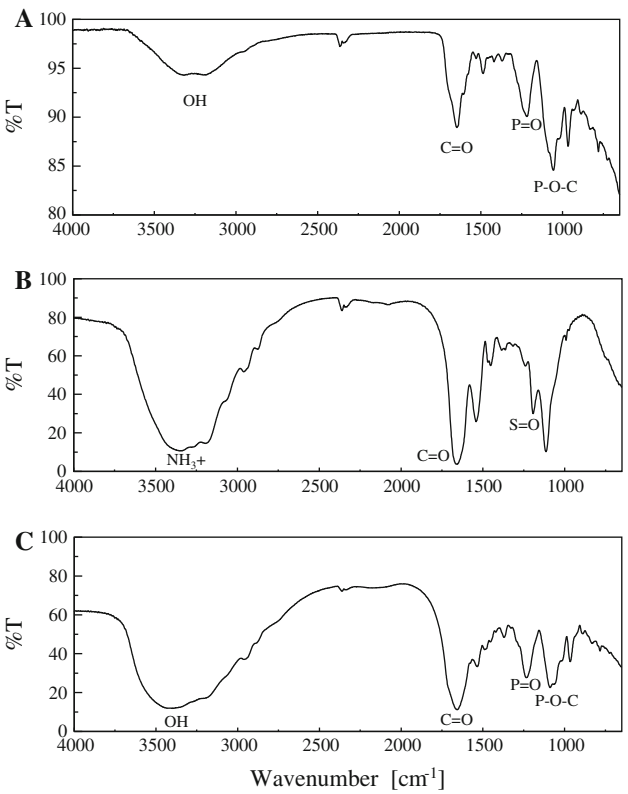

Fig. 2 FT-IR spectra of DNA (**A**), Protamine sulfate (**B**) and DNA/protamine complex powder (**C**)

respectively. Figure 2B shows FT-IR spectrum of protamine sulfate. The broad peak around 3200 cm^{-1} was attributed as NH_3^+ of amino acid salt. The peaks at approximately 1600 and 1190 cm^{-1} could be assigned as C=O, and S=O of organic sulfate salt, respectively. Figure 2C shows FT-IR spectrum of DNA/protamine complex powder after air-drying. The broad peak around 3300 cm^{-1} could be attributed as OH of bound water. The peaks at 1650, 1230, and 1080 cm^{-1} could be assigned as C=O, P=O, and P–O–C stretching modes of DNA/protamine complex, respectively. The peak attributed as S=O of organic sulfate salt stretching modes was not observed. The complex formation could be confirmed.

FT-IR spectra of DNA/protamine complex pastes after air-drying and freeze-drying are shown in Fig. 3. All FT-IR spectra are very similar to each other expect for intensity of the broad peak attributed as OH of bound water. The freeze-dried paste showed greatest decrease in the intensity of the broad peak attributed as OH.

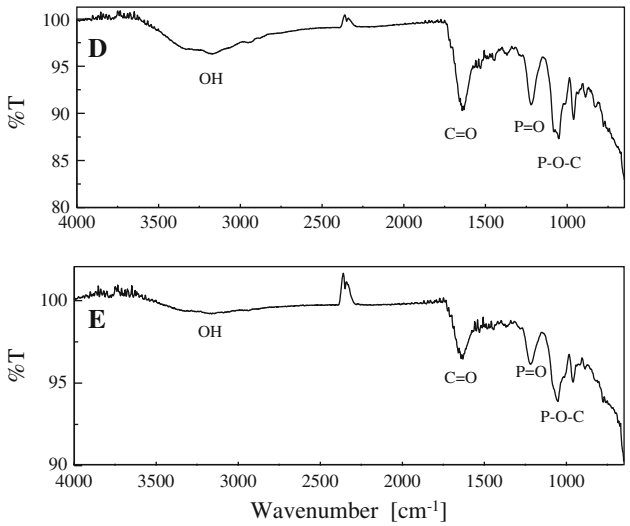

Fig. 3 FT-IR spectra of DNA/protamine complex paste after air-drying for 24 h (**D**), and after freeze-drying for 24 h (**E**)

3.4 Observation of the internal structure of the DNA/protamine complex powder and paste by FE-SEM

Figure 4 shows FE-SEM of the fractured surfaces of the complexes. The porous structure of the DNA/protamine complexes is clearly confirmed. The DNA/protamine complex mass appeared like a honeycomb and areas with pores <5 μm were visible. In contrast, DNA/protamine complex paste appeared to be very different from the powder, and the mean pore sizes of this complex were larger and areas with pores were smaller.

3.5 Viscosity of DNA/protamine complex paste

Figure 5 shows piston stroke-time curves. All curves initially sloped to the right and then gradually showed a plateau. The flow rate (Q) of complex paste was calculated from piston stroke and time in proportional region of a slope on each resulting piston stroke-time curve. Then, the viscosities (η) were calculated according the calculation formula described above. Each viscosity was 167.1, 347.2, and 326.0 Pas, respectively. Mean viscosity and standard deviation were 280.1 and 98.4 Pas.

3.6 Antibacterial sensitivity test

Figure 6 shows the relationship between bacterial growth and time. Two-way ANOVA showed significant differences in antibacterial sensitivity between the control and the complex disks, regardless of types of bacteria used in

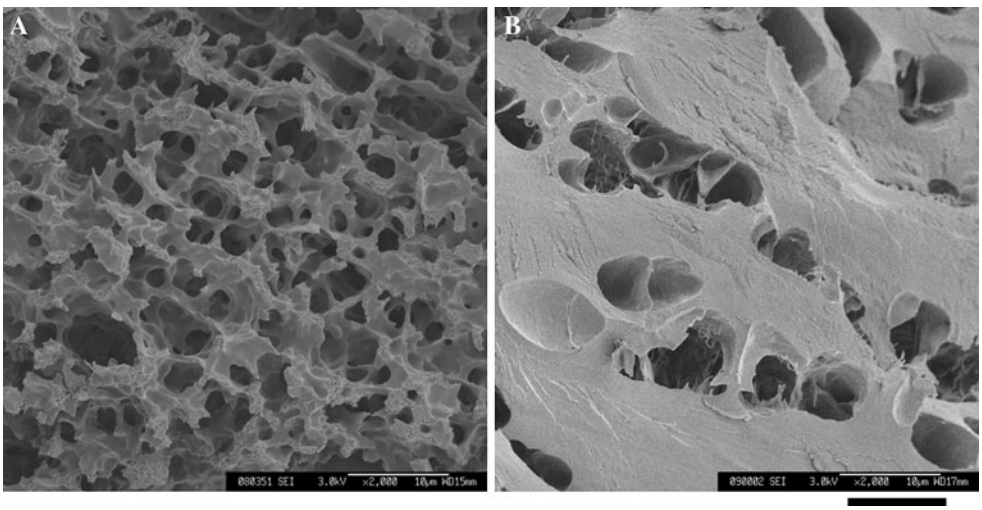

Fig. 4 FE-SEM micrographs of the fracture interior surface of DNA/protamine complexes. (**A**) DNA/Protamine complex powder. (**B**) DNA/Protamine complex paste. *Bar* 10 μm

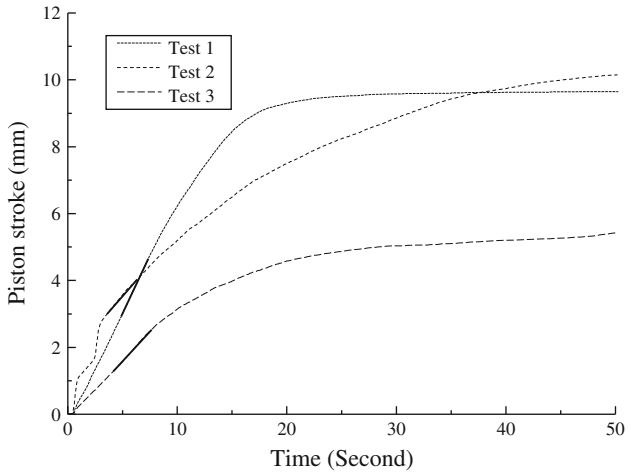

Fig. 5 Piston stroke—time curves. *Solid line*; gradient on each curve used for determining flow rate

this study. However, although differences in bacterial growth rate between control and DNA/protamine complex were observed for limited periods, these differences were very small except for *S. aureus* growth rate. These differences in bacterial growth rate including *S. aureus* growth rate between control and DNA/protamine complex were not observed over limited periods. These results suggested that DNA/protamine complex paste can retard bacterial growth of *S. aureus, P. aeruginosa*, *P. gingivalis* and *P. intermedia* for limited periods.

3.7 Histological changes of implanted sites

Figure 7 shows representative histopathological images of rat soft tissues after the implantation of DNA/protamine complex paste. On day 3 after the surgery, sections of the skin showed remarkable infiltrates of neutrophils in an edematous connective tissue, indicating an acute inflammatory status (Fig. 7A and B). Many fragmented materials were found in the inflamed tissue. There were no infectious changes exhibiting colonies of bacteria or the formation of an abscess in the tissue sections examined. Proliferation of cellular granulation tissue stood out in the tissue sections on day 10 (Fig. 7C). The granulation tissue was composed of numerous fibroblasts and capillaries as well as macrophages. By this period, the materials disappeared from the tissue because macrophages phagocyted those materials (Fig. 7D). Although, a small number of foam-cell-type macrophages were evident in the lesion, the implanted soft tissue was replaced by a dense fibrous connective tissue on day 20 (Fig. 7E and F).

4 Discussion

The clearance of biomaterials in vivo has limited their applicability in therapeutics and tissue engineering [16]. Strategies to reduce clearance in vivo involve fitting biomaterials in tissue defects for the development of new

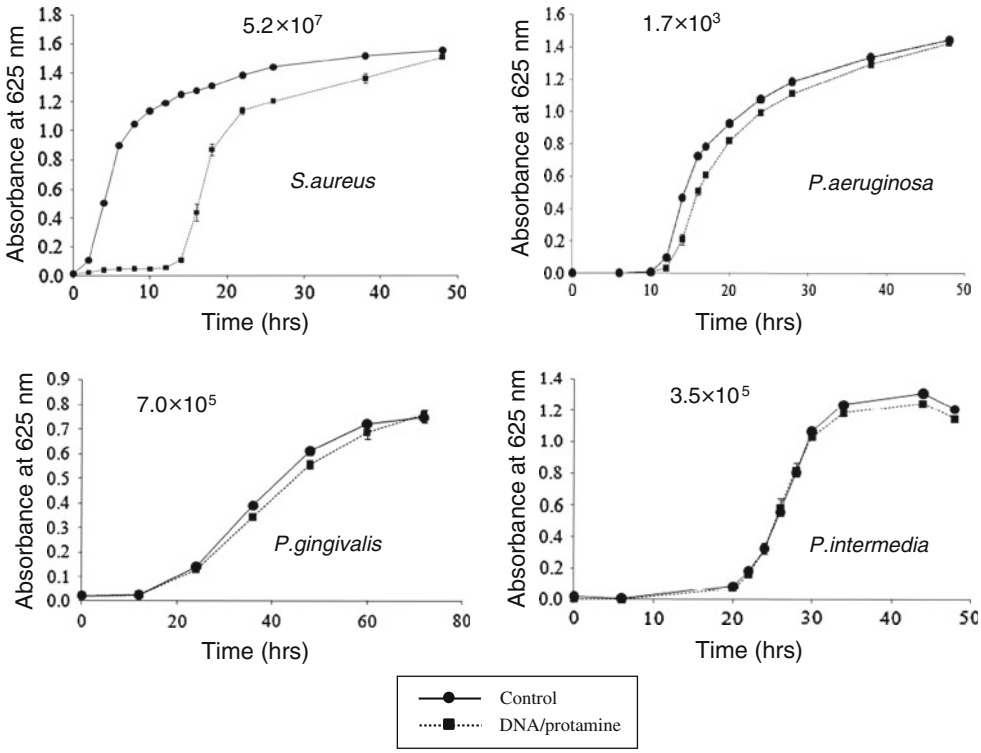

Fig. 6 Inhibitory effect of DNA/protamine complex paste on bacterial growth

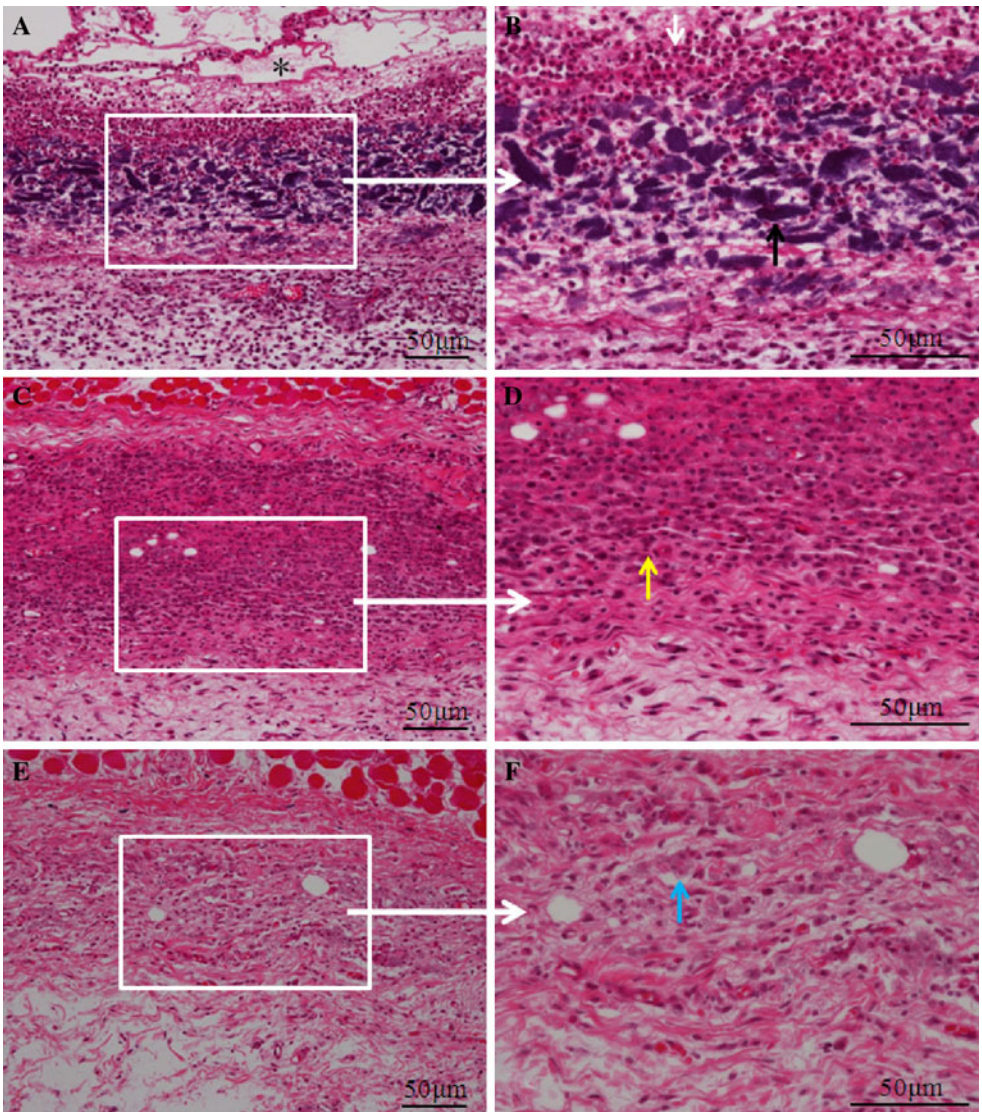

Fig. 7 Histology of soft tissues of skin treated with DNA/protamine complex paste. (**A**) and (**B**) In soft tissue of the skin on day 3, the implant site shows an inflammatory edema (*asterisk*) containing fragmented materials (*black arrow*) and neutrophil infiltration (*white arrow*). (**C**) and (**D**) Cellular granulation tissue on day 10. The tissue contains numerous phagocytic macrophages (*yellow arrow*). (**E**) and (**F**) Replacement of implant site by fibrous connective tissue on day 20. A very few macrophages (*blue arrow*) are noted. Original magnification: ×100 (**A**, **C**, **E**) and ×200 (**B**, **D**, **F**)

biomaterials. Thus, it is considered that paste is one favorable type of biomaterial because it can be easily formed into the desired shape and directly injected with a syringe into tissue defects with various shapes.

DNA/protamine complex powder became a paste by limited mixing with water. It is well known that α-helix protamine lies in the major grooves of DNA, where it neutralizes the negative charge of the phosphate backbone [17]. It is presumed that most protamine binds to grooves of DNA and that, as a result, DNA/protamine complex becomes a linear polymer. Moreover, water is expected to acts as a plasticizer. To elucidate the influence of water, FT-IR measurements were performed. Complex formation between DNA and protamine was clearly confirmed, and the presence of tightly bounded water was also identified. Although, the detailed mechanism is not still clear, the presence of tightly bounded water will be easily combined with added water will be beneficial for acting as a plasticizer. Further detailed study on the mechanism of paste formation will be needed.

The mean viscosity of the DNA/protamine complex paste was very close to that (280.1 Pas) of substrates of dental addition silicon impression materials [18]. Mean flow rate (Q) obtained from piston stroke-time curves was

0.0428 cm^3/s. It means that time for completely filling in 1 cm^3 tissue defect by DNA/protamine paste was about 23 s. The paste could easily pass through a commercial syringe with a needle of 0.25 mm internal diameter shown in Fig. 1. It is expected that the paste can be filled in a tissue defect at more low pressing force or more quickly if a syringe with a needle of more than 0.25 mm internal diameter is used. Moreover, it was found that mold fabrication of DNA/protamine complex paste disks was very easy, as shown in Fig. 4, and that a DNA/protamine complex paste sheet could be easily prepared (not shown). These results suggest that DNA/protamine complex paste will be useful for injectable or sheet biomaterials. For example, we expect that DNA/protamine complex paste can be applied for use as an injectable GBR, as a GTR sheet and as a dressing sheet.

One of the critical factors for new tissue formation is the size of pores in a scaffold, although, there are alternative views on the optimal pore size of a scaffold for cell growth and extracellular matrix production [13–15]. Although, kneading DNA/protamine complex with water reduced its porosity, the mean pore size became larger after kneading (Fig. 4).

In preliminary investigation, it was observed that paste could be easily mixed with inorganic powder such as hydroxyapatite and sodium chloride. It is expected that the biocompatibility or viscosity can be controlled by the addition of apatite. It is also expected that the porosity and pore size of DNA/protamine complex paste can be controlled by a solvent casting/salt leaching technique [19] after the addition of sodium chloride.

Miura et al. [1] reported that protamine showed an antibacterial effect. Johansen et al. [2] reported that protamine can effectively neutralize bacterial cell surface charges and may lead to conformational change and disruption of the cell membrane; protamine from salmon killed growing gram-positive bacteria and significantly inhibited the growth of gram-negative bacteria. Therefore, it is expected that the DNA/protamine complex paste will show antibacterial effect by releasing protamine from the complex. As shown in Fig. 6, the DNA/protamine complex paste delay the growth speed of all bacteria compared with the control (Teflon disk) for limited periods. DNA/protamine complex paste has antibacterial effects against gram-positive bacteria same as protamine alone.

Histological changes consist of three sequential phases: (1) acute inflammatory reaction, (2) proliferation of inflammatory granulation tissue containing numerous phagocytic macrophages, and (3) replacement of fibrous connective tissue. These findings are identical to those shown as typical biological reactions against biomaterials implanted into the skin [11, 12]. However, in contrast to typical histological evidence showing the formation of foreign-body granuloma against the implanted biomaterials, the lesion in the present study lacks histological signs of granuloma formation, such as infiltrates of multinucleated giant cells and encapsulation by dense fibrous connective tissue. Thus, it suggests that the DNA/protamine complex paste may be more susceptible to biodegradation than DNA/chitosan complex [11].

5 Conclusions

Porous DNA/protamine complex became a paste by kneading with water, which could then pass through a needle of 0.25 mm internal diameter. The DNA/protamine complex paste has suitable viscosity for clinical use. Generally, paste can easily mix with inorganic and organic materials such as hydroxyapatite, cytokine and antibacterial agent, etc. Moreover, the DNA/protamine complex paste showed a mild soft tissue response. Therefore, DNA/protamine complex paste with biodegradable property will be useful as injectable biomaterials with a syringe for the repair of defects of living tissue, GBR treatment and/or GTR treatment in dentistry.

Acknowledgment This study was supported in part by Grants-in-aid for Scientific Research (B) (19390505 and 23390455) and by a Grant-in-aid for strategic study base formation support business (S1001059) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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