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Biological and Chemical Assessment of DNA/Chitosan Complex Film

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Abstract: Transparent DNA/chitosan complex film was prepared from DNA/chitosan complex powder via hydrothermal hot pressing. In this study, we investigated the binding of daunorubicin hydrochloride (DH), proteins adsorption (fibronectin and albumin), mineral deposition, and rat soft-tissue response to the DNA/chitosan complex film to determine whether this film has potential for use in membranes for bone tissue engineering. The binding ratio of DH to DNA/chitosan complex film was very close to the excision number of 3.7 for the binding of DH by native DNA. This indicated that DNA in the complex did not denature as a result of hot pressing. Apatite was formed on the surface of the film after immersion in simulated body fluid. However, quartz crystal microbalance analysis showed that protein absorption to the DNA/chitosan complex was very low. It was found that this film has an affinity for minerals rather than proteins. Although the film remained in an almost unchanged configuration at 21 days after subcutaneous implantation, the response of rat soft tissues to the film was mild. These results suggest that DNA/chitosan complex film has potential for use as a membrane in bone tissue engineering.

Key words: DNA/chitosan complex, Hydrothermal hot-pressing, Protein binding, Biodegradation, Protective membrane

Introduction

Deoxyribonucleic acid (DNA) has suitable properties for use in biodegradable biomaterials such as a scaffolds and drug delivery systems. For example, DNA has phosphate groups, which have high affinity for calcium ions, and it is less antigenic than other macromolecules such as proteins and polysaccharides. However, it is difficult for native DNA alone to be used as a biomaterial because it is a water-soluble polymer. To address this limitation, Fukushima *et al.*¹⁻³⁾ prepared cationic DNA/poly-L-amino acids [poly-L-arginine (poly-arg), poly-L-lysine (poly-lys) and poly-L-histidine (poly-his)] and DNA/chitosan complexes, which were water insoluble, for the application of DNA as a new biodegradable medical material. It was reported that the complexes had no serious cytotoxicity and showed only a mild soft-tissue response in rats in the course of biodegradation.

Segura *et al.*⁴⁾ suggested that the clearance of scaffold materials

in vivo has limited their application for tissue engineering even if they have advantageous biological properties as biomaterials. Thus, it is considered that the formability of biomaterials is very important for fitting into tissue defects during implantation.

Fukushima *et al.*⁵⁾ reported that DNA/chitosan complexes could be mold-fabricated by rinsing methods with PBS buffer, whereas DNA/poly-L-amino acids cationic complexes showed poor formability. However, Irie *et al.*⁶⁾ reported that DNA-chitosan, DNA/poly-arg and DNA/poly-lys complex powders turned into transparent films by use of hydrothermal hot-pressing methods. They also suggested that these complexes could be useful for clinical treatments requiring thin membranes or films, such as protective membranes for stomatitis and incised oral wounds.

In bone tissue engineering, mixtures of inorganic biomaterials such as hydroxyapatite with blood or saline are filled into bone defects. However, these mixtures are excluded from bone defects if the barrier of the bone defect is not sufficient. The membranes used as a barrier and an overcoat are required to stay with the mixtures being used to repair the bone defects. The biodegradation

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rate of these membranes is very important for bone tissue engineering, because new bone formation may take more than 3 months after implantation. Thus, we considered that membranes with fast biodegradation rates are unsuitable as bone substitutes.

Irie et al.⁶⁾ described in their study with DNA/chitosan and cationic poly-L-amino acids complex films that DNA/chitosan film was biodegraded within 90 days after implantation, whereas the other films biodegraded within 14 days. Thus, we considered that DNA/chitosan films could be a candidate membrane for use in bone tissue engineering, and we investigated whether this was the case.

The hydrothermal hot-pressing method heated the DNA/chitosan complexes at 80 °C with mechanical loading (30 MPa) during film processing⁶⁾. It is not yet clear whether heating denatures DNA in the DNA/chitosan complex or not.

In this study, we firstly investigate the denaturation of DNA in DNA/chitosan films by a daunorubicin hydrochloride (DH) binding method, and secondly, investigated the apatite-forming behavior by immersing the film in simulated body fluid. We also examined the binding of various proteins to the film by quartz crystal microbalance (QCM) analysis. Finally, we explored the histological responses of rat soft tissues to the film.

Materials and Methods

Preparation of DNA/chitosan complexes

Salmon testes DNA (Maruha Nichiro Corporation, Tokyo, Japan), which has been cleaved with nuclease into 300-bp fragments, was used in this study. Chitosan (500 mg; average molecular weight = 129,000; Maruha Nichiro Corporation, Tokyo, Japan) was dissolved in 40 ml of 0.2 N HCl. NaOH solution (0.2 N) and distilled water were added gradually to the chitosan to bring the pH to 5.0 and the volume to 100 ml.

DNA (500 mg) was dissolved in 100 ml of distilled water and then added to the chitosan solution. The mixture was stirred at 20 °C for 1 h. The DNA/chitosan complex was collected by centrifugation at 9,000 rpm for 10 min and washed with distilled water, after which the DNA/chitosan complex was freeze-dried (FD-5, Eyela, Tokyo, Japan).

Preparation of DNA/chitosan complex film

Firstly, DNA/chitosan complex powder (100 mg) and distilled water (50 µl) were mixed, and DNA/chitosan complex powder disks with distilled water were prepared by the same method as described previously⁶⁾.

The fabricated disk (14 mm diameter and 1 mm height) was placed onto the surface of a PTFE sheet (50 mm × 50 mm, 0.3 mm thickness) and then the another PTFE sheet was placed on the top surface of complex disk. The DNA/chitosan complex disk sandwiched by PTFE sheets was placed on the surface of a polyethylene terephthalate plate (100 mm × 100 mm, 1.0 mm

thickness), which was placed onto the metal disk in the hydrothermal hot pressing machine (IMC-180C, Imoto Machinery Co., Ltd, Kyoto, Japan) and then another polyethylene terephthalate plate was placed on top of the PTFE sheet. The processing of the DNA/chitosan film was carried out by pressing the disk at 30 MPa at 80 °C for 10 min. After being cooled at room temperature, the film was trimmed and used for the following experiments.

Intercalation and groove binding of daunorubicin hydrochloride within DNA/chitosan film

DNA/chitosan film (20 mg) was immersed for 48 h at 20 °C in 5 ml of 1.4×10^{-3} mol/l DH (Wako Chemicals, Osaka, Japan) aqueous solution. The absorbance from 400 to 600 nm of each solution was measured before and after the reaction with a V-560 spectrophotometer (Jasco Corp., Tokyo, Japan). The amounts of intercalated and bound DH in the films 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.

Apatite formation on the film

The DNA/chitosan film (10 mm × 10 mm, 30 µm thickness) was immersed in 20 ml of simulated body fluid (Hank's solution)⁷⁾ and stored at 37 °C for 3 and 21 days. The simulated body fluid was renewed weekly for the 21-day study period. The surface of each sample was observed using a field emission scanning electron microscope (FE-SEM; JSM-6330F, JEOL, Tokyo, Japan) after immersion. The crystallographic structure of the crystals formed on the DNA/chitosan film was analyzed by means of X-ray diffraction (XRD; Ultima 4, Rigaku, Tokyo, Japan) with an X-ray source of CuKα and a power of 44 kV × 50 mA.

Quartz crystal microbalance analysis of protein binding to the film

A QCM with a resonant frequency of 27 MHz (Affinix Q4, Initium Inc., Tokyo, Japan) and biotinylated DNA (20 bp, 1 µM; Fasmac Co., Ltd., Atsugi, Japan) were used to measure the reaction amount of albumin (100 µg/ml; Sigma, St. Louis, USA) and fibronectin (100 µg/ml; Roche, Basel, Switzerland) to the DNA/chitosan complex. Biotinylated DNA was immobilized on the Au electrode sensor surface of the QCM in accordance with methods described by Hayakawa et al.⁸⁾.

The DNA-immobilized sensor was exposed to 500 µl of TE buffer (containing 0.15 mol/l NaCl). When the frequency of the sensor had stabilized, chitosan solution (100 µg/ml, Nacalai Tesque, Kyoto, Japan) was injected, and the frequency was monitored as a function of time. The binding amount of chitosan to DNA was determined from the frequency shift. Next, the sensor surface with DNA/chitosan complex was exposed to 500 µl of

Figure 1. Schematic illustration of DNA/chitosan complex (a). Transparent complex film fabricated by hydrothermal hot pressing (b).

Figure 2. The amount of daunorubicin hydrochloride released from DNA/chitosan complex film.

Figure 3. Field emission scanning electron micrographs of the DNA/chitosan complex film immersed in simulated body fluid for 3 days (a) and 21 days (b).

fresh TE buffer containing 0.15 mol/l NaCl. After stabilizing the frequency, aqueous solutions (3 μl) of albumin or fibronectin were injected. The amounts of albumin and fibronectin binding to the DNA/chitosan complex were determined from the frequency shift. At 27 MHz, a frequency shift of 1 Hz corresponded to a mass change of an approximately 0.62 ng/cm². All experiments were performed in triplicate.

Implantation and histological evaluation

Animal experiments (No. 07002) were performed in accordance with the ethical guidelines for animal experiments of Fukuoka Dental College. The studies were conducted using 6-week-old male Sprague-Dawley rats weighing approximately 200 g. Surgery was performed under general anesthesia induced by 2 % isoflurane (Wako Chemicals, Osaka, Japan) and air mixture gas flowing at 1.0 l/min using an anesthesia gas machine (SF-B01, MR Technology Inc., Tsukuba, Ibaraki, Japan). An incision was made in the back of the rats, and the complex film (10 mm × 10 mm) was implanted subcutaneously. After the insertion of samples, the soft tissues were closed in separate layers by suturing with intracutaneously resorbable Vicryl 3-0 (Ethicon Inc.,

Somerville, USA). The control was a group of sham-operated rats that did not undergo sample implantation. The rats (20 in total) were divided into two groups (one experimental group and one control group) for observation times of 14 and 21 days, respectively. Five rats were used for each group at each time point. At each time point, the animals were sacrificed by injection of an overdose of isoflurane. After sacrifice, the subcutaneous tissues containing the implanted film were excised immediately. They were fixed in 4 % paraformaldehyde in phosphate buffer (pH 7.4), dehydrated with a graded alcohol series, cleared in xylene, and embedded in paraffin using routine procedures. The specimens were sectioned at 3 μm. The sections were stained with hematoxylin and eosin for histological observation using a light microscope (Eclipse 55i, Nikon, Tokyo, Japan).

Results

Preparation of DNA/chitosan films

A schematic illustration of the presumed structure of DNA/chitosan complexes and the transparent complex film is shown in Fig. 1. The thickness of the DNA/chitosan complex film was approximately 30 μm.

Figure 4. The XRD pattern of precipitates on the surface of the DNA/chitosan film after immersed in simulated body fluid (HA: hydroxyl apatite)

Intercalation and groove binding of daunorubicin hydrochloride within DNA-chitosan films

The cumulative amounts of intercalated and bound DH in the films are shown in Fig. 2. The amounts of intercalated and bound

Figure 6. Histology of subcutaneous tissues of skin treated with the DNA/chitosan complex film after 14 days (a) and 21 days (b). Arrows indicate resorptive fossae of the film.

DH in the films increased with increasing immersion time within 24 hours after immersion and then plateaued. The colorless, transparent films changed to red, transparent films after immersion in DH solution. The amount of intercalated and groove-bound DH in the films was about 2.40 mg after immersion for 144 h. These results suggested that one molecule of DH intercalated and groove-bound to approximately 3.5 bp of DNA.

Apatite formation on the film

Fig. 3 shows FE-SEM pictures of the DNA/chitosan film immersed in simulated body fluid for 3 and 21 days. Scanning electron microscopic observations revealed precipitate formation on the film. In the early stage (3 days' immersion), and globular precipitates were scattered over the surface of the film. At 21 days' immersion, the film was covered with larger amounts of globular

Figure 5. Frequency shift after albumin (a) and fibronectin (b) binding to biotinylated DNA/chitosan complex to a quartz crystal microbalance sensor. Arrows indicate a second injection of albumin or fibronectin.

precipitates. The amount of precipitate increased with immersion time.

Fig. 4 shows the XRD pattern of precipitates on the surface of the DNA/chitosan film after immersion. The XRD pattern shows diffraction lines characteristics of hydroxyapatite.

QCM analysis of protein binding to the film

The amount of DNA immobilized on the surface of Au in the QCM sensor averaged 349.0 ng/cm², and the amount of chitosan electrically reacted to immobilized DNA on the sensors averaged 249.2 ng/cm² (data not shown).

Fig. 5 shows the frequency shift of the QCM sensor after the binding of albumin or fibronectin to the biotinylated DNA/chitosan complex on QCM sensor. After injection of albumin or fibronectin, a decrease in frequency begun but later the changes in frequency

were very small. Moreover, the frequency shift showed a plateau after the second injection (arrows) of albumin or fibronectin. The binding amounts of albumin or fibronectin to the biotinylated DNA/chitosan complex were 75.6 ng/cm² and 52.7 ng/cm², respectively.

Implantation and histological evaluation

Fig. 6 shows representative histological images of rat subcutaneous tissues after implantation of the DNA/chitosan film. The DNA/chitosan complex film remained almost fully intact at 14 days after implantation. Twenty-one days after implantation, a small amount of fragmented film was scattered in the implanted site. Multinuclear giant cells were noted in the adjacent portion of the implanted film on day 21 (Fig. 6b), and resided in the resorptive fossae of the film. These giant cells showed a phagocytic activity against the DNA/chitosan complex film. Fragmentation into small pieces and degradation of the complex film was not shown 21 days after implantation.

Discussion

In this study, we prepared a DNA/chitosan complex film from DNA/chitosan, powder via hydrothermal hot pressing in accordance with a previous study⁶⁾. Similar transparent DNA/chitosan complex film to that in previous study was obtained. Although this film was prepared at 80°C, the effect of this temperature on denaturation of DNA in the complex is still unknown. Fukushima *et al.*²⁾ reported that DH intercalated with and bound to the groove of the DNA/chitosan complex, indicating that DNA in the complex maintained its double-stranded helical structure. Thus, we measured the amount of DH bound to the film in this study. A 144 h-immersion resulted in the binding of one molecule of DH to approximately 3.5 bp of DNA in the film. This is very close to the excision number of 3.7 for the binding of DH by native DNA⁹⁾. This result indicated that heating to 80 °C did not denature DNA in the complex. It was also suggested that antibacterial activity could be generated by intercalating and groove binding of antibacterial materials such as acriflavine to the film.

Transparency is advantage for observation during convalescence because the wound is visible directly through the DNA/chitosan complex film. Moreover, it is considered that this film can be placed easily in narrow spaces such as spaces between alveolar bone and oral epithelium because this film is very thin.

Kawazoe *et al.*¹⁰⁾ reported that mesenchymal stem cells adhered and spread on the complex membranes at DNA/chitosan prepared at a DNA/chitosan ratio 1:1. However, the DNA/chitosan complex film showed poor dermal fibroblast cell attachment, with cells forming spheroids in spite of preparing the complex at a DNA/chitosan ratio of 1:1 in the previous study⁶⁾. It is well known that cells can attach to areas where proteins such as fibronectin,

collagen, etc. had absorbed¹¹⁻¹³⁾. Thus, we investigated the adsorption of fibronectin and albumin to films by QCM in this study. The level of adsorption of fibronectin and albumin to the films was very low (Fig. 5). Furuya *et al.*¹⁴⁾ reported that approximately 440 ng/cm² of fibronectin absorbed on titanium (titanium sensor of QCM apparatus). The absorbed amount of fibronectin detected in the present study (75.6 ng/cm²) was approximately 17 % of the absorbed amount reported by Furuya *et al.* Because many factors, including electrostatic interaction, hydrogen bonding and hydrophobic interaction¹⁵⁻¹⁸⁾, influence the adsorption of proteins onto biomaterials, the detailed mechanism of low protein adsorption is still unclear. We presumed that low protein adsorption affected poor dermal fibroblast cell attachment, resulting in cell spheroid formation. From these results, it may be expected that this film could be used as a membrane where adhesion needs to be prevented. Adhesion prevention is an advantage for a membrane because the film can be removed easily from the implantation area when retreatment is required, such as due to infection after implanting.

Unlike proteins, this film has good affinity for minerals such as a calcium compounds. As shown in Fig. 3, precipitated apatite formation on the surface of the film was observed within 3 days in simulated body fluid, and larger amounts of apatite were observed on the film after 21 days' immersion. These results corresponded with the results of immersion tests with the disk-shaped DNA/chitosan complex in a previous study¹⁹⁾. We considered that DNA/chitosan complex film will be useful as a membrane for bone tissue engineering.

Application of such films greatly depends on the biodegradation rate *in vivo*. Lu *et al.*²⁰⁾ reported that degradation behavior is important for tissue-engineered cell/polymer construction involving cell growth and tissue regeneration. Nair and Laurencin²¹⁾ described that the degradation rate of materials should match the intended application and should not be higher than new matrix synthesis *in vivo*. In the case of this DNA/chitosan complex film used as a membrane for a bone tissues engineering, the film is required to keep almost to its original form for more than 3 months *in vivo* because new bone formation needs more than 3 months. In a previous study, it was found that most of films with 30µm thickness biodegraded within 90 days after implantation in soft tissues of rat. However, we considered that increasing the thickness of the film could slow the rate of biodegradation.

For biodegradable biomaterials, tissue response is very important during biodegradation. As shown as Fig. 5, the DNA/chitosan complex film remained almost intact at 21 days after subcutaneous implantation and tissue responses were mild. Histological observations showed that macrophages and multinucleated giant cells actively phagocytosed the DNA/chitosan complex film at 21 days after subcutaneous implantation. It is

well known that chitosan-decomposing enzyme are not present in humans²²⁾. Thus, we considered that the biodegradation of DNA/chitosan complex film is mainly dependent on phagocytosis by macrophages and multinucleated giant cells.

In conclusion, transparent DNA/chitosan complex film of 30 μm thickness was prepared from DNA/chitosan complex powder via hydrothermal hot pressing. The binding ratio of DH to the DNA/chitosan complex film was very close to the excision number of 3.7 for the binding of DH by native DNA. This indicated that DNA in the complex did not denature during hot pressing. The DNA/chitosan complex film showed potential of apatite formation on the film after immersion in simulated body fluid within 3 days. From QCM analysis, low adsorption of fibronectin and albumin to the film was revealed. It was found that this film has an affinity for minerals rather than proteins. Although the complex film remained almost intact after 21 days' subcutaneous implantation, the response of rat soft tissues to the film was mild. Therefore, it is expected that DNA/chitosan complex film has potential for use as a membrane in bone tissue engineering.

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