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Research paper

Feasibility of simple chitosan sheet as drug delivery carrier

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Abstract

Chitosan, a biodegradable and biocompatible polysaccharide, is a potentially useful material in various fields. We developed a simple chitosan sheet and examined the possibility of using an adriamycin-containing chitosan sheet as a drug carrier for controlled release. To prepare a carrier consisting only of chitosan, a chitosan suspension was subjected to acid-alkaline treatment, mixed with adriamycin, frozen and freeze-dried. The adriamycin-containing chitosan sheet was inserted into the peritoneal cavity of mice in order to investigate its biodegradation. The appearance of decomposition of chitosan was observed using scanning electron microscopy, and adriamycin in urine and liver was detected for 1 and 2 weeks, respectively. Adriamycin metabolites were detected in plasma for 2 weeks. Furthermore, adriamycin remained in the chitosan sheet without being metabolized after 2 months. These results suggested that the chitosan sheet prepared in this study might improve therapeutic efficacy in topical lesions as a carrier of sustained-release drugs.

Keywords: Chitosan; Drug carrier; Adriamycin; Biodegradation; Biomaterials; Controlled release

1. Introduction

Chitosan, a biodegradable polysaccharide, comprising glucosamine and *N*-acetylglucosamine, is an alkaline-deacetylated chitin derived from the exoskeletons of insects and shells of crustaceans. Chitin and chitosan have been used in a wide variety of biomedical applications [1–7] since Prudden et al. reported the acceleration of wound healing by chitin and chitosan. In addition to its physiological activity [8–10], chitin and chitosan are biocompatible and biodegradable. Thus, the medical applications of chitin and chitosan have recently been the subjects of numerous studies.

Several carriers derived from chitosan have been developed as drug delivery systems [11–15]; however, the clinical applications remain under investigation. A wound dressing developed commercially, which is employed externally, has been shown to be clinically effective [4]. However, there was concern regarding the side effects induced by an excessive immune reaction to the ingredients contained in the biomedical product; chitosan is digested by activated macrophages. If a chitosan carrier containing drugs can be applied to the focuses of a disease, drugs can be released gradually and improve therapeutic efficiency. In order to exert notable drug activity in topical lesions without any side effects induced by biomedical materials, the quality and form of the material is very important. In the present study, we used a simple technique to develop a pure chitosan sheet with a flexible flat shape, and furthermore investigated chitosan biodegradation and subsequent drug (adriamycin; ADM) release from the sheet in order to explore the feasibility of chitosan as a drug carrier in topical lesions.

2. Materials and methods

2.1. Chemicals

Chitosan (PSH 80, Mw. 5×10^5–1×10^6, 80% deacetylated), and ADM metabolites (adriamycinol and adriamycinon) were kind gifts from Yaizu Fishery Co. (Shizuoka, Japan) and Pharmacia (Tokyo, Japan), respectively.

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ADM was purchased from Kyowa Hakko Co., Ltd. (Tokyo, Japan) and other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

2.2. Preparation of chitosan sheets

Chitosan (1% w/v) was dissolved in hydrochloric acid (0.05 N), with agitation, overnight at room temperature, and sodium hydroxide (0.05 N) was added to neutralize the chitosan solution, which was then gelled. The gelled solution was filtered with a glass filter (25G P5), washed with distilled water several times, and homogenized using a homogenizer (KIINEMATICA, PT 10-35, Switzerland) after the addition of distilled water up to give a 1% chitosan concentration. Homogenized gelling solution (20 ml) was cooled gradually to −20 °C and was then maintained for over 12 h in a plastic schale (ϕ 9 cm). Chitosan sheet thickness was dependent on the volume of the solution. The frozen solution was lyophilized and the chitosan sheet was cut into suitable shapes before the experiment. Sheet permeability to water and air was 0.8–10 $cm^3/cm^2/h$ (measured using water column at room temperature) and 2×10^3–8×10^3 $cm^3/cm^2/min$ (measured with air pressure of 10–15 kgf/cm^2 at room temperature), respectively.

2.3. Animal experiments

Female ddY mice (age; 5–6 weeks) were obtained from SRL (Shizuoka, Japan) and housed 5 or 1 per cage, with a stainless steel feeder under standard conditions with a 12-h light/dark cycle. Animals were allowed free access to water and standard diet. To investigate the biodegradation of chitosan sheets *in vivo*, a piece of circular sheet (13 mg, 5–10 mm) was placed in the peritoneal cavity of mouse, and was subsequently removed for observation by scanning electron microscopy. To measure ADM metabolism, urine was collected daily and mice were sacrificed for liver and blood samples after sheet placement in the cavity. The experimental protocols were conducted in accordance with the Guidelines for Animal Experiments at the University of Shizuoka.

2.4. Scanning electron microscopy

Scanning electron microscopy (SEM) was performed using a Hitachi S-2500 (Tokyo, Japan). Sheets removed from the peritoneal cavity were dried using a critical-point drying technique, following treatment with 50%, 70%, 90% and 100% ethanol and immersion in ethanol–isoamyl acetate (1:1). Dried samples were Pt–Pd coated before SEM observation.

2.5. Preparation of adriamycin-containing chitosan sheet

Adriamycin (ADM) was added at 1 mg per 100 ml of the homogenized gelling solution described in Section 2.2, and this solution was mixed for 1 h at room temperature. The solution was frozen at −20 °C for over 12 h in a plastic schale (ϕ 9 cm), and thawed. The watery chitosan sheet was washed with distilled water several times to remove excess ADM, and was then lyophilized.

2.6. ADM determination

ADM concentration was measured by modifying the reported high-performance liquid chromatography (HPLC) method [16]. An HPLC column with a fluorescence detector (Hitachi F1050, Tokyo, Japan) was used for analysis. A normal phase column (Mightysil Si60, Kanto Kagaku, Tokyo, Japan) was used as the stationary phase. The mobile phase consisted of chloroform–isopropanol–acetic acid–water–sodium acetate buffer (100:100:14:14:1, pH 4.5) at a flow rate of 1.0 ml/min. Fluorescence signals were monitored at Ex 470 nm and Em 585 nm. ADM was extracted by chloroform/methanol (4:1) and was then centrifuged at 15,000 rpm for 15 min. The phase-separated chloroform/methanol layer was analyzed by HPLC. Pure ADM was used as a standard. The content of ADM adsorbed by the chitosan sheets was estimated to be 1 μg/mg dry chitosan. Fluorescence microscopy was used to confirm the adsorption of ADM.

3. Results

3.1. Morphological characteristics of chitosan sheet

The structure of chitosan sheet as observed by microscopy is shown in Fig. 1. The sheet was symmetric, and on SEM, was found to consist of multiple porous layers and an uneven surface with apertures (Fig. 1A and B). The shape of the pores was not uniform, with the diameter varying between 50 and 500 μm. Under a phase-contrast microscope, the sheet appeared to have a knitted stitch and fine fibrous structure (Fig. 1C). The presence of uneven apertures is reflected in the difference between the light and dark regions on the micrograph. These structures enabled the permeation of water and air. The texture of the sheet was smooth and flexible, similar to a sponge or gauze, and would thus be easy to handle in medical applications. In addition, chitosan sheet with adsorbed ADM was observed by both SEM and phase-contrast microscopy, and no significant changes in morphological characteristics were noted (data not shown).

3.2. Biodegradation of an ADM-containing chitosan sheet in mouse

An ADM-containing chitosan sheet was inserted into the peritoneal cavity of a mouse for several days in order to observe its biodegradation. Fig. 2 shows SEM images of the surface structure of the chitosan sheet after biodegradation for 7 days, as initial stage of the degradation was seen at 24 h. At 7 days, the surface was considerably more irregular and the difference between light and dark regions

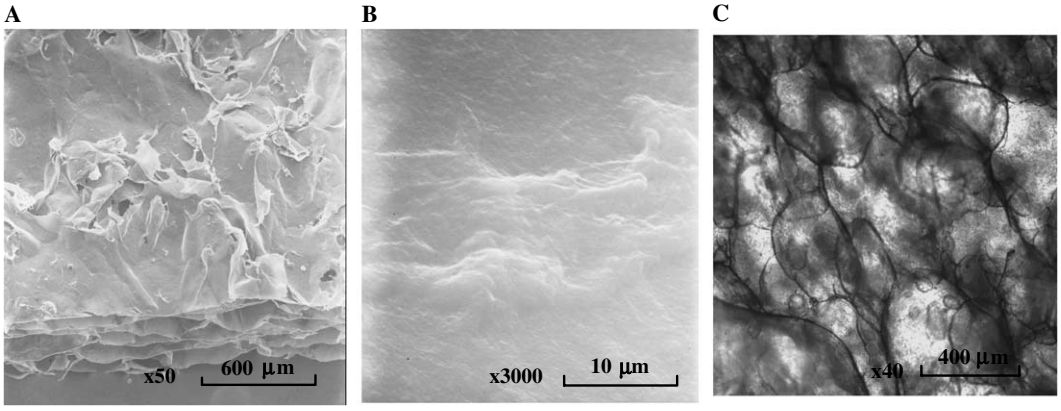

Fig. 1. Photomicrographs of chitosan sheet. (A and B) scanning electron microscopy, (C) a phase-contrast microscopy.

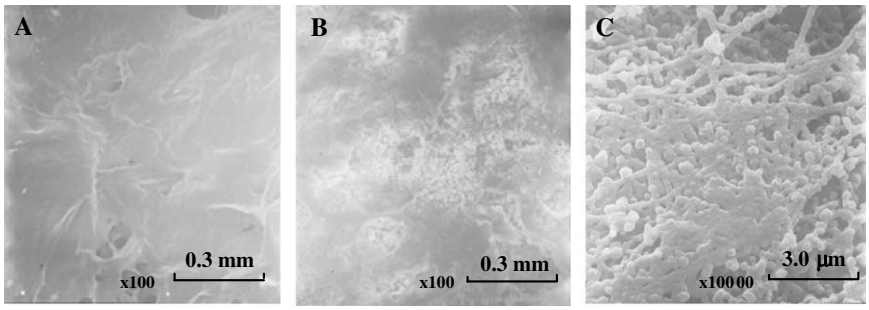

Fig. 2. Photomicrographs (scanning electron microscope) of adriamycin-containing chitosan sheet after biodegradation in mouse peritoneal cavity for 7 days. (A) Before biodegradation, (B and C) after biodegradation.

was clearer than with the original, which appears to be a series of small cracks at 100× (Fig. 2B). Furthermore, biodegradation of macromolecular substances entangled in the fibers was observed both at the surface and within the sheet at 10,000× (Fig. 2C). The sheet gradually decomposed and became very fragile, and for several months a lump of particulates was visible; its appearance was substantially different from the original sheet, providing a visual indication of the chitosan's degradation.

3.3. Feasibility of ADM-containing chitosan sheet as drug carrier

ADM released from the sheet was measured in liver, blood and urine using HPLC (Fig. 3). ADM was detected in urine and liver for 1 and 2 weeks, respectively, after the ADM-containing chitosan sheet was placed in the peritoneal cavity. In contrast, no ADM was detected in blood; however, ADM metabolites (adriamycinol and adriamycinone) were released into blood for 2 weeks. In particular, adriamycinone exhibited steady release from 1 to 2 weeks, before subsequently decreasing, In addition, the residual ADM on chitosan sheet was determined after application in vivo (Fig. 4A). The ADM content of the chitosan sheets decreased gradually, which indicates that it was released into tissues. ADM was released from chitosan sheet embedded in the abdominal cavity. Furthermore, besides its metabolites, over 0.5 μg/g of adriamycin remained in the chitosan sheet without being metabolized after 2 months in vivo (Fig. 4B), showing that the chitosan sheet might maintain and exhibit the medicinal effects of adsorbed drugs for several months.

4. Discussion

We developed a drug carrier that was composed of 100% chitosan that was able to adsorb ADM and was subsequently biodegraded in mice. Chitosan is a slightly cationic natural polysaccharide with numerous reactive amino groups in its molecular structure that possesses many useful characteristics; viscosity, antibacterial activity, humectant, biocompatibility, biodegradation and physiological activity in animals. Various products derived from chitosan have been developed in a variety of fields, and products with useful biological properties attract the attention of medical fields. However, most such products are not produced from 100% chitosan, but rather contain chemical modifications or included impurities, which may adversely influence the living body. The chitosan sheet in this study was produced using a simple technique based on agglutination (described in Section 2).

Chitosan was originally used as a flocculant to coagulate negatively charged suspended particles founded in turbid natural water [17] and as a water-purifying agent to remove heavy metals in wastewater treatment [18,19]. Chitosan is also a known sorbent, effective in the sorption of transition

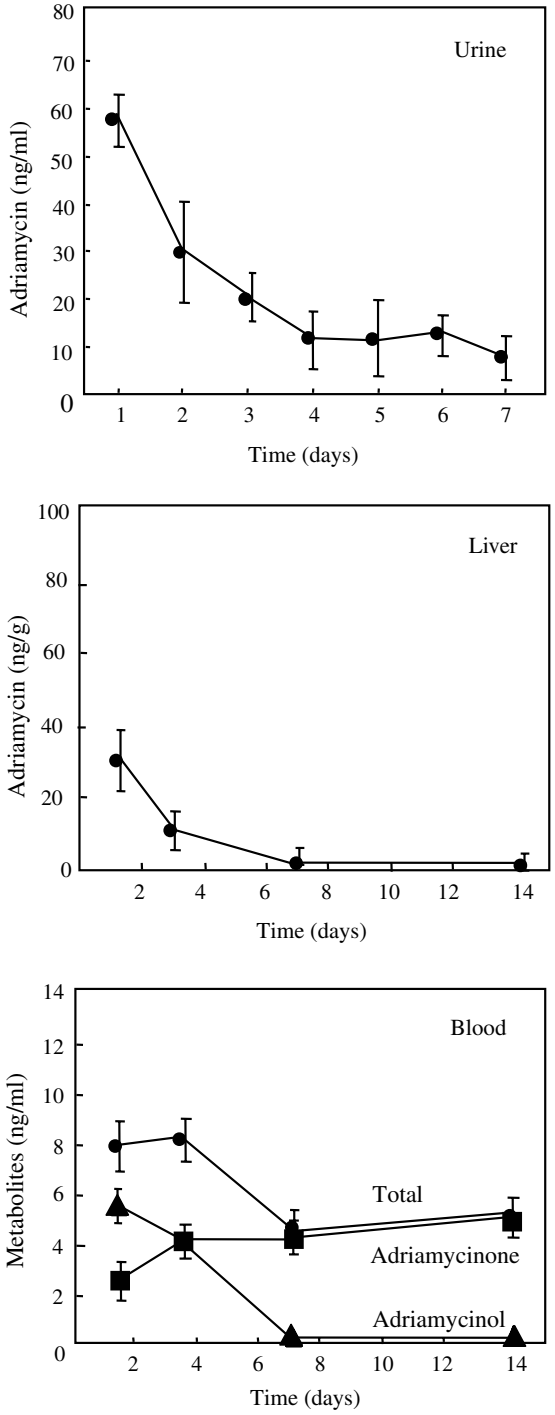

Fig. 3. Release kinetics of adriamycin and its metabolites from chitosan sheet in mice. Data are presented as means ± SD ($n = 4$).

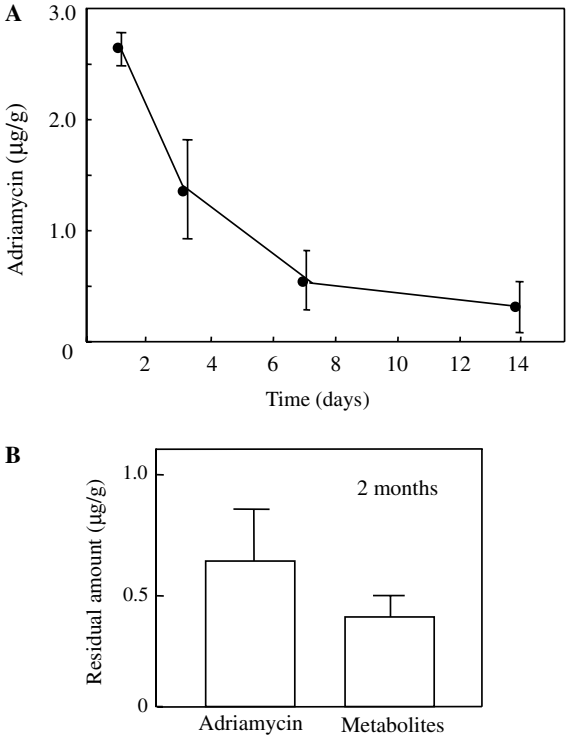

Fig. 4. Decline of adriamycin in the chitosan sheet after the application to abdominal cavity of mice. (A) Residual adriamycin for 2 weeks, (B) residual adriamycin and its metabolites after 2 months. Data are presented as means ± SD ($n = 4$).

metal ions because the amino and hydroxyl groups on the chitosan chains can serve as coordination and electrostatic interaction sites [20–22]. Chitosan is probably dominated simultaneously by adsorption, ion exchange and chelation. These characteristics may enable chitosan to adsorb ADM and coagulate uniformly under freezing conditions.

Fluorescence microscopy confirmed ADM adsorption by chitosan, even though the sheet was washed firmly with water. There were no significant differences between the ADM-containing chitosan sheet and non-ADM-containing chitosan sheet on SEM observation. In addition, no differences in biodegradability were seen between the two, which suggests that the presence of ADM did not affect chitosan sheet conformation (data not shown). However, the conformation of the chitosan molecules in the sheet is unknown, both in the presence and absence of ADM. Further experiments on the chemical behavior of chitosan molecules with ADM are necessary for subsequent drug design and clinical applications.

The biodegradation images shown in Fig. 2 have the same appearance as those obtained after degradation by lysozyme (data not shown), showing that the decomposition of the sheet in mice is mediated by lysozyme, which is known to be ubiquitous in the body and to play an important role in degrading chitosan into oligomers that are further hydrolyzed into simple sugars *in vivo* [23]. Lysozyme release from specific cells or organs into blood is induced by chitosan. The enhancement of lysozyme is probably a biological defensive function, followed by activation of the macrophage system in animals [24]. We observed that cells like macrophage adhered to the surface of the chitosan sheet after application (data not shown). The chitosan sheet was stable in water and degraded with lysozyme or an acidic solution in vitro, and ADM was extracted from the sheet with an organic solvent (chloroform/methanol), however, ADM was not detected from the sheet that reacted only with lysozyme in vitro. These

results suggest that biodegradation of the chitosan sheet that releases ADM in vivo is mediated not only by lysozyme but also by biofunctions related to metabolism, which might be affected by the interaction between ADM and the chitosan molecules. Although we were not certain of the rate of ADM release and the mechanism of degradation of chitosan, the biodegradable chitosan sheet appeared to decompose and release ADM. We know this because ADM was detected in liver and urine for 1 and 2 weeks, respectively. In addition, ADM metabolites, adriamycinone, and adriamycinol, were present in the blood for 2 weeks; these were measured by HPLC as shown in Fig. 3. The half-life of ADM in blood is so short that it disappears within several hours. It is difficult to detect actual ADM in blood; however its metabolites exhibit medicinal effects *in vivo* [25]. For ADM, metabolites are effective in medical treatment; however, it is necessary to further investigate drugs that are not readily metabolized and their effectiveness in direct application using in chitosan sheets.

The ADM content of the chitosan sheets embedded in the abdominal cavity decreased in time dependency as shown in Fig. 4, and it appeared that ADM was released into tissues as a result of the degradation of the chitosan sheet. The sheet decomposed and became very fragile, and particulates were visible after several months. Furthermore, over 1 μg/g of adriamycin and its metabolites still remained in the sheet even though the appearance of the sheet was disfigured after 2 months in vivo. This illustrates that the chitosan sheet was able to release drugs without being ruined.

In addition, several studies have demonstrated the high biocompatibility, low immunogenicity and biodegradability of chitosan, suggesting that chitosan can be considered as a sustained drug release material, as controlling degradation is possible by utilizing the biodegradability properties.

Multi-porous beads prepared from chitosan have been tested for physical and immunological properties as an anticancer chemotherapeutic carrier, but antitumor activity was found to be less than that of free ADM [26]. Recently, 100% chitosan particles containing a drug were developed [1,2], but were not efficacious in the affected region because mobility prevented continuous of drug activity during medical treatment. Furthermore, small particles with drugs may be digested by macrophages before exerting sufficient therapeutic effects, even if macrophages are activated. With regard to therapeutic efficiency, when producing a chitosan drug carrier for controlled release, material quality and form, in addition to function, must be carefully considered. A non-woven fabric of 100% chitosan is suitable for preventing undesirable side effects, such as inflammation and allergies.

In conclusion, the chitosan sheet developed in this study is a suitable material for medical use. This sheet material is permeable to water and air, and is flexible, in addition to possessing the properties typically associated with chitosan. The present study suggests the feasibility of the present chitosan sheet as a drug carrier and more advanced clinical applications are expected in both animals and humans.

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