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Research paper

Injectable in situ forming drug delivery system for cancer chemotherapy using a novel tissue adhesive: Characterization and in vitro evaluation

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Abstract

Injectable polymers that are biocompatible and biodegradable are important biomaterials for drug delivery system (DDS) and tissue engineering. We have already developed novel tissue adhesives consisting of biomacromolecules and organic acid derivatives with active ester groups. The resulting tissue adhesive forms in situ as a gel and has high bonding strength for living tissue as well as it has good biocompatibility and biodegradability. Here, we report on the physicochemical properties and in vitro evaluation of this novel tissue adhesive consisting of human serum albumin (HSA) and tartaric acid derivative (TAD) containing doxorubicin hydrochloride (DOX). The results of the measurement of physicochemical characteristics indicate that the gelation time and gel strength of HSA–TAD gels can be controlled according to the material composition. The bonding strength of HSA–TAD adhesives was found to be sufficient to adhere at focus and to correspond with the cross-linking density of HSA–TAD gels. Furthermore, the release of DOX from HSA–TAD gels was sustained for approximately 100 h in an in vitro evaluation. The novel tissue adhesive, therefore, is expected to be applicable for use as an injectable in situ forming DDS.

Keywords: Tissue adhesive; Hydrogel; Drug delivery; Human serum albumin; Tartaric acid; Injectable; In situ forming; *N*-Hydroxysuccinimide; Doxorubicin

1. Introduction

Injectable polymers that have biocompatibility and biodegradability are important biomaterials for drug delivery system (DDS) and tissue engineering. Multiple synthetic and natural biodegradable polymers have been investigated for these purposes, including polyesters, polyethers, poly amino-acids, polysaccharides, and proteins [1]. These polymers are employed as injectable DDS, and especially as injectable DDS for cancer chemotherapy, and have been investigated actively so as to minimize the toxic side effects and increase the carcinostatic pharmaceutical effects [2]. Methods of local administration of DDS, nanoparticles [3–5], microspheres [6,7], polymeric micelles [8–10], liposomes [11–14], and hydrogel systems [15,16] for targeting and controlled release have been investigated with non- and biodegradable polymers. However, the targeting DDS has not been satisfactorily achieved. Accordingly, the injectable in situ forming DDS with hydrogel system, that demonstrates a sol–gel transition in a physiological environment, has many uses [17,18]. For example, water-soluble polymers with stimuli-responsiveness have been widely used as injectable DDS, e.g., oxidized alginate–gelatin hydrogel [19], thermoresponsive (poly(*N*-isopropylacrylamide)-g-gelatin) [20], poly(ethylene oxide)-

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poly(prolylene oxide)-poly(ethylene oxide) [21] and poly(ethylenoxide)-poly(L-lactic acid)-urethane-poly(L-lactic acid)-poly(ethylene oxide) [22].

Furthermore, in situ gelling formulations derived from biopolymers that mimic the extracellular matrix such as collagen, gelatin, and hyaluronic acid are notable because biopolymers are generally more biocompatible and biodegradable than synthetic polymers. These polymers are generally cross-linked by noncovalent bonds such as ion bonds, electrostatic and hydrophobic interactions, or covalent bonds using cross-linking reagents such as glutaraldehyde and condensing agents such as carbodiimide [23]. For clinical usage, it is important that the in situ gelling formulations combine measurable stability and good biocompatibility. While noncovalent bonds are unstable in the body, cross-linking reagents have high toxicity.

We have recently developed novel tissue adhesives consisting of biomolecules such as collagen, gelatin, and human serum albumin, and organic acid derivatives with active ester groups [24–28]. These tissue adhesives are synthetically superior to commercially available surgical glues such as fibrin glue [29] and biomacromolecule–aldehyde glue [30] with regard to both bonding strength and biocompatibility. Furthermore, the novel tissue adhesive possesses the potential to be a carrier of injectable in situ forming DDS. An injectable in situ forming DDS using fibrin glue has been already reported, although it was not achieved for the long-term release of drugs [31–33].

We report herein the preparation of an injectable in situ forming DDS using human serum albumin (HSA) [34] and tartaric acid derivative (TAD) for cancer chemotherapy. Carcinostatic doxorubicin hydrochloride (DOX) was employed in this preparation because it is widely active for several solid tumors, including malignant lymphoma, hepatoma, carcinoma of the breast, lung, stomach, cholecystis, and pancreas, and osteosarcoma [35,36]. In the present study, we evaluated and demonstrated the physicochemical characteristics and the in vitro release behavior of HSA–TAD in in situ forming DDS.

2. Materials

Human serum albumin was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Tartaric acid, *N*-hydroxysuccinimide (HOSu) and doxorubicin hydrochloride were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dicyclohexylcarbodiimide (DCC) was purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). All other reagents used were of HPLC or analytical grade without further purification.

3. Methods

3.1. Synthesis of TAD

TAD was prepared by a procedure similar to that previously reported [24]. Briefly, tartaric acid (5 g) was first dissolved in THF (200 ml), and then HOSu (9.58 g) and DCC were added. After mixing for 30 min, the mixture was concentrated by rotary evaporation under reduced pressure to remove the THF. The resulting mixture was recrystallized to yield pure TAD, which was confirmed by ^{1}H NMR spectroscopy and elemental analysis.

3.2. Determination of gelation time and gel strength

HSA was dissolved in PBS (0.1 M, pH 7.4) at various concentrations. HSA solutions were weighed to 0.5 g in a polypropylene tube, and different amounts of TAD (0.05, 0.075 and 0.1 mmol/0.8 g of HSA sol.) were added to the HSA solution and stirred vigorously. In the case of the HSA–TAD containing DOX, DOX was added to the HSA solution before the addition of TAD. In order to measure the gel strength of HSA–TAD, disk-shaped gels ($\varphi 20.0 \times 1.0$ mm) were prepared using a mold made of silicone rubber spacer and two glass plates. Using a rheometer (Haake RS1 Rheometer; Thermo Electron, Dreieich, Germany) at 37 °C the gelation time and gel strength were determined as the intersection point of G′ (storage modulus) and G″ (loss modulus), respectively.

3.3. Measurement of swelling ratio

Disk-shaped HSA–TAD gels ($\varphi 20.0 \times 1.0$ mm) were immersed in PBS (0.1 M, pH 7.4) maintained at 37 °C. The weight of the swollen HSA–TAD gels (Ws) was periodically measured until they were completely swollen. The weight of the dried HSA–TAD gels (Wd) was then determined after lyophilization. The swelling ratio was calculated by the following equation:

$$\text{Swelling ratio} = (W_s - W_d)/W_d \quad (1)$$

3.4. Measurement of bonding strength

The bonding strength of HSA–TAD tissue adhesive was measured by the following procedures as shown in Fig. 1. A piece of collagen casing was fixed on each of two pieces of PET film. Onto one of them was dropped 70 μL of HSA–TAD tissue adhesive. Then it was stuck to the other collagen casing fixed on PET film. The bonding area was fixed at 10 mm × 10 mm. After 10 min at 37 °C, the bonding strength was measured, using a tensile testing machine (TA-XT2i, EKO INSTRUMENTS Co., Ltd., Tokyo, Japan), as the shearing bonding strength. BOLHEAL® (fibrin-based blue; KAKETSUKEN, Kumamoto, Japan) was also used as a control. Three samples were tested to measure the same bonding strength ($n = 3$).

3.5. Determination of DOX

The amount of DOX in the release medium was determined by RF-HPLC. The HPLC system consisted of an HPLC pump (PU-2080 plus), an intelligent autosampler

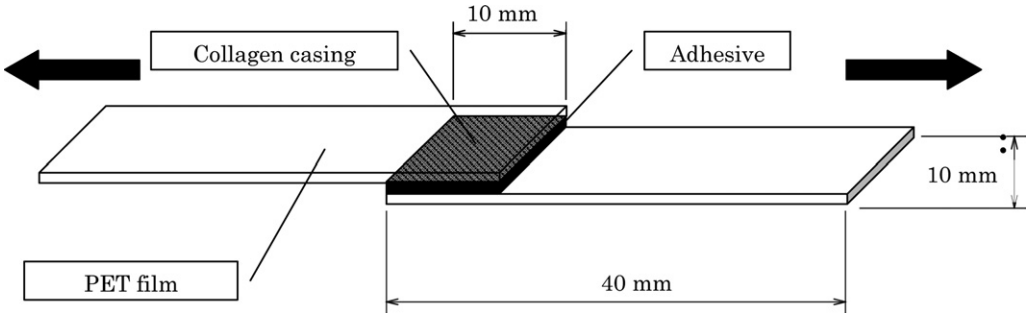

Fig. 1. Schematic of the samples to measure bonding strength.

(AS-2055 plus), a fluorescence detector (FP-2020 plus), column ovens (CO-2060), and a degasser (DG-2080-53); all of which were purchased from JASCO Inc. (Tokyo, Japan). The separation was carried out using a reverse-phase C_{18} column (COSMOSIL, Nacalai Tesque, Inc. Japan). The mobile phase was prepared as a mixed solvent (water/acetonitrile/acetic acid = 77:22:1), and was delivered isocratically at a flow rate of 1 ml/min. The effluents were monitored fluorometrically using an excitation wavelength (λ_{ex}) of 470 nm and an emission wavelength (λ_{em}) of 585 nm.

3.6. Stability test of DOX under in vitro release conditions

The stability test of DOX in the release medium was performed using 0.5 μM-DOX in 0.1 M-PBS (pH 7.4) solution. DOX solution was incubated at 37 °C in a polypropylene tube, and the amount of DOX was periodically measured by RF-HPLC.

3.7. In vitro release study

The in vitro release of DOX from HSA–TAD gels was carried out by the following procedure: the HSA–TAD gels (φ10.0 × 5.0 mm) were prepared by a method similar to the one described in Section 3.2, and kept in 100 ml of 0.1 M-PBS (pH 7.4) at 37 °C. One-half milliliter of the release medium was collected periodically and replenished with 0.5 ml of fresh PBS. The amount of DOX in the release medium was determined by RF-HPLC.

4. Results and discussion

4.1. Characterization of TAD

Tartaric acid (TA) is a dihydroxy derivate of dicarboxylic acid. The synthesis of TAD (Fig. 2) was carried out by the standard protocol using HOSu and DCC [37]. Characterization of TAD was performed by ^{1}H NMR and elemental analysis, that is, by a comparison of the integral ratio of the peaks at 2.8 and 4.8 ppm, which are assigned as methine and succinimydyl groups, respectively. The elemental analysis (C, H, N; wt%) of TAD shows C, 41.87; H, 3.51; N, 8.14. Calculated values are C, 41.87; H, 3.51; N, 8.14. It is demonstrated that highly purified TAD can be obtained with high yield (approximately 60%).

4.2. Physicochemical properties of HSA–TAD gels

In order to inject locally with a syringe pump, adjustment of the gelation time is very important. As shown in Fig. 3, the gelation time of HSA–TAD gels depended on the HSA and TAD concentrations. That of HSA–TAD gels (35 w/w%–0.1 mmol/0.8 g of HSA sol.) was approximately 26 s, and the time lengthened with decreasing HSA and TAD concentrations. It was supposed that the gelation time depends on the number of cross-linking points, that is, the amide creates a bond between active ester groups in TAD and amino groups of Lys residues in HSA. Furthermore, the gelation time was lengthened

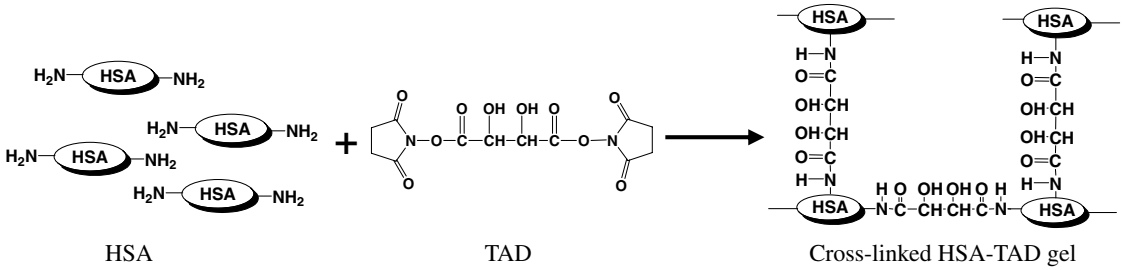

Fig. 2. Cross-linking reaction of HSA with TAD.

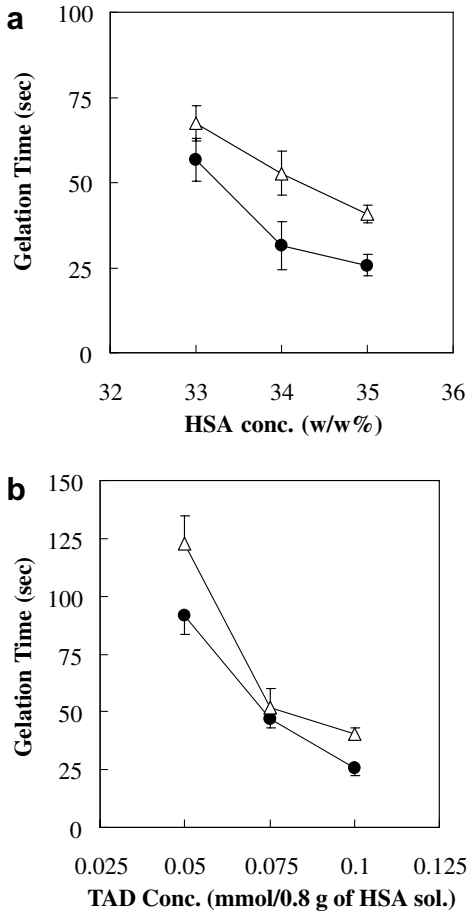

Fig. 3. Dependence of HSA (a) and TAD (b) concentrations on gelation time with (●) and without (△) DOX. Error bars exhibit standard deviations ($n = 3$).

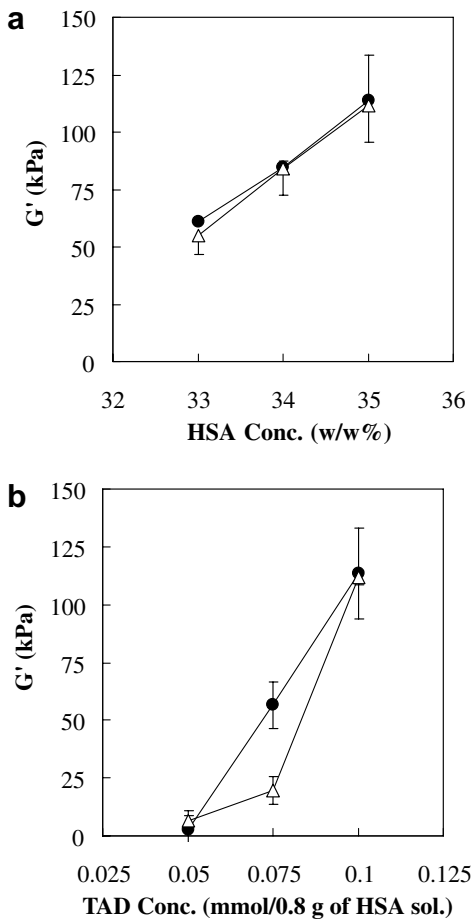

Fig. 4. Dependence of HSA (a) and TAD (b) concentrations on gel strength with (●) and without (△) DOX. Error bars exhibit standard deviations ($n = 3$).

by the addition of DOX because DOX has an amino group in the molecule that can react with the active ester group of TAD. In addition, the lowering of the pH of the HSA and TAD mixture triggered by free HOSu during the cross-linking reaction caused an increase in the gelation time. These results indicate that the gelation time can be controlled by the composition ratio of TAD and HSA.

Measurement of the gel strength of HSA–TAD gels with/without DOX was also performed as shown in Fig. 4. The gel strength of HSA–TAD gel increased with increase in HSA and TAD concentrations. The concentrations of HSA and TAD had been varied from 33 to 35 w/w%, and from 0.05 to 0.1 mmol/0.8 g of HSA sol., respectively. The bonding and gel strength of HSA–TAD generally depended on the cross-linking density. The active ester groups of TAD are likely to be inactivated by hydrolysis before amide bond formation at low HSA concentrations. Therefore, the cross-linking density decreases with the decrease in HSA concentrations, despite there being adequate amino groups. Furthermore, only the gelation time of HSA–TAD decreased by the addition of DOX. HSA–TAD gels were prepared using enough amino groups for the active ester groups. If all amino groups in HSA completely react with active ester groups of TAD, the cross-linking density of the resulting HSA–TAD gel would be decreased with the addition of DOX. Therefore, these results suggest that not all amino groups derived from Lys residues in HSA uniformly react with active ester groups of TAD. The conformational location of Lys residues in HSA is very important to the reaction with TAD, because HSA is globular protein. Amino groups of Lys residues located on the surface of HSA molecules might be likely to react and form an amide bond. It is expected that the final cross-linking density be equal to the gel with/without DOX, because the number of surface amino groups of HSA is less than that of the active ester groups of TAD.

The swelling ratio of HSA–TAD gels was also determined using Eq. (1), as shown in Fig. 5. The obtained ratios indicated a behavior similar to that of the bonding strength and gel strength in relation to HSA and TAD concentrations. Therefore, the results suggest that the swelling ratio also depends on the cross-linking density of the gel; in other words, the swelling ratio is the reciprocal of the cross-linking density.

The cross-linking density (v_e; mol/cm^3) of HSA–TAD gels was calculated from the Flory–Rehner equation (Eq. (2)) [38],

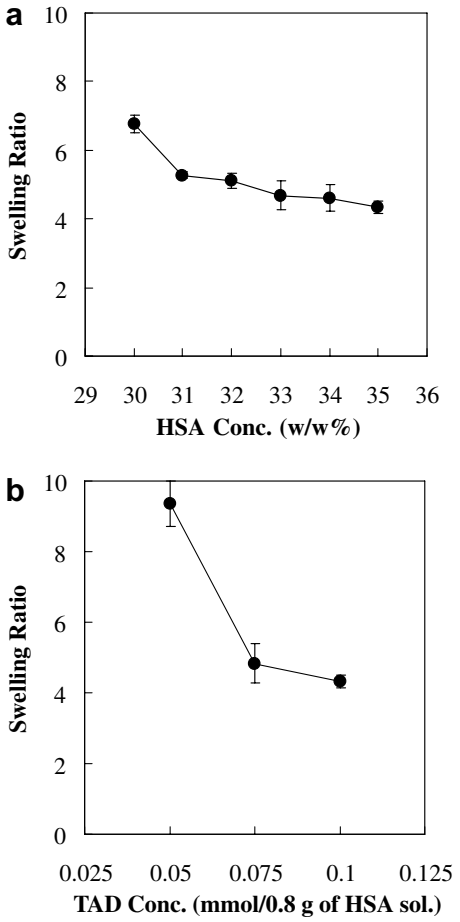

Fig. 5. Dependence of HSA (a) and TAD (b) concentrations on the swelling ratio without DOX. ((a) HSA concentrations were varied from 30 to 35 w/w% at a fixed TAD concentration of 0.1 mmol/0.8 g of HSA sol. (b) TAD concentrations were 0.05, 0.075, and 0.1 mmol/0.8 g of HSA sol. at a fixed HSA concentration of 35 w/w%.) Error bars exhibit standard deviations ($n = 3$).

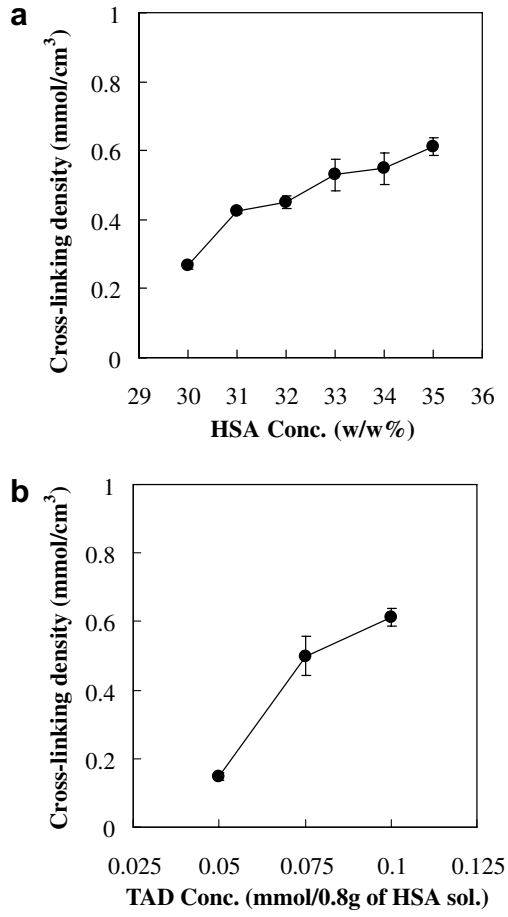

Fig. 6. Dependence of HSA (a) and TAD (b) concentrations on the cross-linking density of HSA–TAD gels without DOX. ((a) HSA concentrations were varied from 30 to 35 w/w% at a fixed TAD concentration of 0.1 mmol/0.8 g of HSA sol. (b) TAD concentrations were 0.05, 0.075, and 0.1 mmol/0.8 g of HSA sol. at a fixed HSA concentration of 35 w/w%.) Error bars exhibit standard deviations ($n = 3$).

$$v_e = -[\ln(1 - v_2) + v_2 + \chi_1 v_2^2][v_2^{1/3} - 2v_2/f]^{-1} \quad (2)$$

and

$$v_2 = (1/\rho_p)[(SR/\rho_s) + (1/\rho_p)] - 1 \quad (3)$$

where χ_1 is the Flory–Huggins interaction parameter, f is the cross-linking functionality = 2, V_1 is the molar volume of solvent = 18.062 (cm^3/mol) for water, ρ_p is the polymer density = 1.364 (g/cm^3) for HSA [34], ρ_s is the density of the water at 37 °C = 0.993 (g/cm^3), and SR is the swelling ratio, as shown in Fig. 6. χ_1 was assumed to be 0.35 which has been previously used to model a similar interaction [39,40]. The cross-linking density of HSA–TAD gels increased with increase in HSA and TAD concentrations. These results demonstrated that physicochemical properties of HSA–TAD depend on the cross-linking density.

4.3. Bonding strength of DOX-containing tissue adhesive

As shown in Fig. 7, the bonding strength of HSA–TAD with/without DOX was measured as the tensile-shearing adhesive strength using test pieces. The bonding strength was gradually increased with increasing HSA concentrations at a fixed TAD concentration of 0.1 mmol/0.8 g of HSA sol., that is, 123.6 ± 2.4 kPa, 156.4 ± 7.1 kPa, and 171.7 ± 9.7 kPa at HSA 33, 34, and 35 w/w%, respectively. It has been demonstrated that the bonding strength increases with increase in the cross-linking density of HSA–TAD gels with/without DOX. Furthermore, the bonding strength did not change in response to the addition of DOX. This result suggests that DOX does not affect the cross-linking density of the resulting HSA–TAD gels.

A comparison of the bonding strength between HSA–TAD tissue adhesive (HSA 33 w%–TAD 0.1 mmol/0.8 g of HSA sol.) with DOX and fibrin-based glue was also carried out. As shown in Fig. 8, the bonding strength of HSA–TAD tissue adhesive was found to be 14 times stronger than that of fibrin-based glue.

These results indicate that HSA–TAD adhesive with DOX has good bonding strength for use as injectable in situ forming DDS.

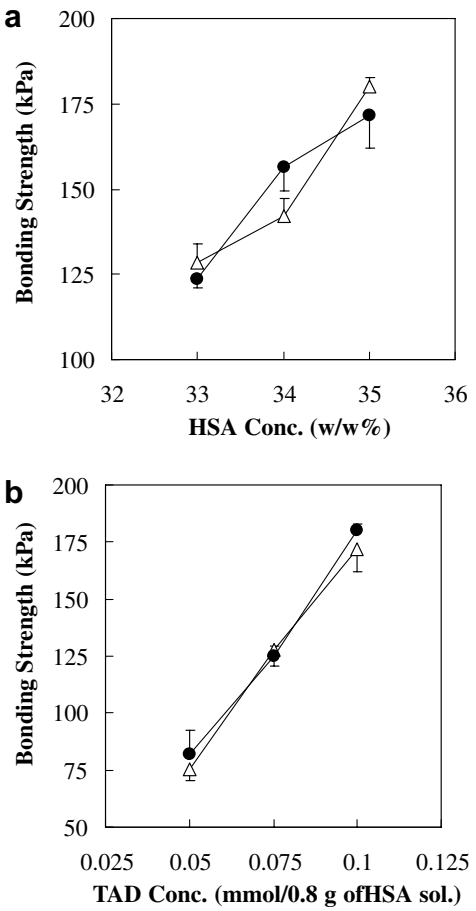

Fig. 7. Dependence of HSA (a) and TAD (b) concentrations on bonding strength with (●) and without (△) DOX. Error bars exhibit standard deviations ($n = 3$).

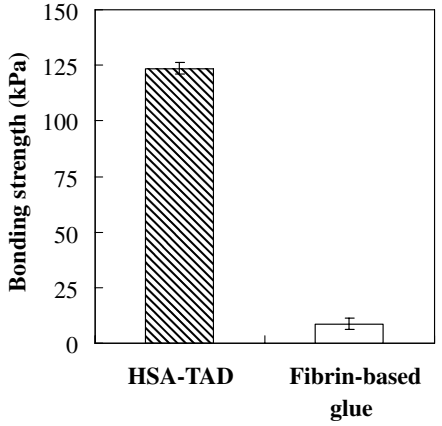

Fig. 8. Comparison of the bonding strength between HSA–TAD tissue adhesive with DOX (HSA 33 w%–TAD 0.1 mmol/0.8 g of HSA sol.) and fibrin-based glue. Error bars exhibit standard deviations ($n = 3$).

4.4. In vitro release of DOX from HSA–TAD gels

It is known that DOX in the solid state is stable even under high temperature; however, it becomes unstable once dissolved in a solvent [41]. The stability of DOX in solution may depend on several factors that include temperature, pH value, buffer concentration and so on. It has been demonstrated that DOX is stable in acidic solution (pH 3.0–6.5), but that it rapidly degrades at high pH (pH 6.5–12) [42]. It is therefore necessary to evaluate the stability of DOX in the medium. The degradation profiles of DOX in the release medium (0.1 M-PBS, pH 7.4) at 37 °C are shown in Fig. 9. Seventy percent of the DOX remained after approximately 20 h. These results show that the half life of DOX under this condition is approximately 48 h, and therefore that DOX is unstable in 0.1 M-PBS (pH 7.4) at 37 °C.

The in vitro release of DOX from HSA–TAD gels consisting of various concentrations of HSA and TAD was carried out (Fig. 10). The released amounts of DOX from HSA–TAD gels decreased with increase in the cross-linking density of HSA–TAD gels. This behavior corresponds to the swelling ratio of HSA–TAD gels. The amount of DOX released from all HSA–TAD gels reached a maximum after 24 h, and then decreased with time. In comparison with DOX solution alone, impregnation of HSA–TAD gel with DOX clearly increased the stability of DOX. These behaviors can be explained as follows: DOX existing on the surface of HSA–TAD gel was initially released within approximately 24 h. Following this, DOX release from the bulk gel then occurred over approximately 100 h. In order to achieve the long-term release of DOX, it is important to stably retain DOX in HSA–TAD gel via molecular interactions. In our case, DOX was immobilized in the HSA–TAD gel by electrostatic interactions between the amino groups of DOX and the carboxyl group of Glu and Asp residues in HSA. Formation of an amide bond also occurred between DOX and TAD. Furthermore, it should be noted that DOX in HSA–TAD gel is more stable than it is in solution, because the pH in HSA–TAD gel is decreased by the formation of free-HOSu after a cross-linking reaction. Although the free-HOSu is released from HSA–TAD gel after injection, it is known that HOSu has

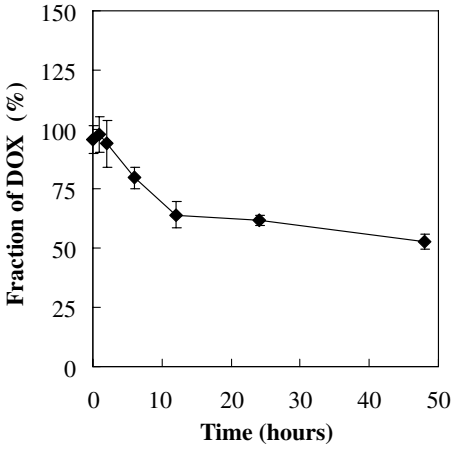

Fig. 9. The stability of DOX in the release medium (0.1 M-PBS, pH 7.4) at 37 °C. Error bars exhibit standard deviations ($n = 3$).

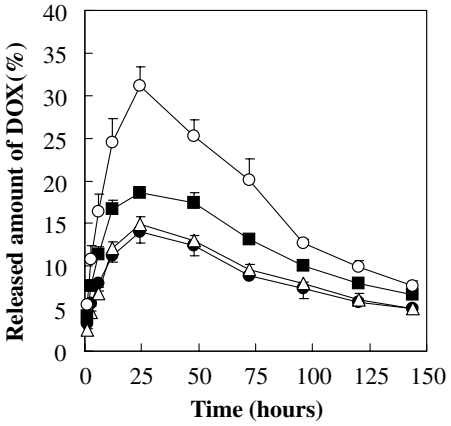

Fig. 10. In vitro release profiles of DOX from HSA–TAD gels consisting of 33 w/w% HSA (●), 34 w/w% HSA (△), 35 w/w% HSA (■) with 0.1 mmol/0.8 g of HSA sol. of TAD, and 35 w/w% HSA (○) with 0.075 mmol/0.8 g of HSA sol. of TAD, respectively. Error bars exhibit standard deviations ($n = 3$).

no mutagenic activity [43]. These results therefore suggest that HSA–TAD gels have appropriate characteristics to act as carrier for DOX, and that HSA–TAD gels containing DOX possess biocompatibility.

5. Conclusion

We have shown here that a novel injectable DDS can release and stabilize DOX. The release rate of DOX from HSA–TAD gel can be controlled by the matrix composition. The release of DOX from HSA–TAD gels reached a maximum after 24 hours, after which the concentrations were maintained for a long period, approximately 100 h. These results suggest that this novel tissue adhesive can possibly be applied as an injectable in situ forming DDS for cancer chemotherapy. In vivo study of this novel injectable DDS is now in progress.

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