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European Journal of Pharmaceutics and Biopharmaceutics 67 (2007) 676–681
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Antitumor effect of an injectable in-situ forming drug delivery system composed of a novel tissue adhesive containing doxorubicin hydrochloride

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Received 10 January 2007; accepted in revised form 24 March 2007
Available online 31 March 2007

Abstract

Our group has developed a novel tissue adhesive composed of biomacromolecules and organic acid derivatives which have good biocompatibility and exhibit high bonding strength to living tissues. We propose to use this tissue adhesive for in-situ forming drug delivery system (DDS) for cancer chemotherapy. In a previous work, we had prepared a novel in-situ forming DDS composed of human serum albumin (HSA) and tartaric acid derivative (TAD) containing doxorubicin hydrochloride (DOX), and we had demonstrated an in vitro release profile of DOX from HSA–TAD gel for approximately up to 100 h. Here, we report on antitumor effect of this injectable in-situ forming DDS. Local injection of DOX by the HSA–TAD was administered to human colon carcinoma (WiDr) implanted subcutaneously onto the immunodeficient mouse. The results of the in vivo experiments showed that the presence of DOX in blood of mice was detectable for up to 3 days, and that the tumor volume was effectively minimized with injection of HSA–TAD containing DOX. The in-situ forming DDS with the novel tissue adhesive containing DOX, therefore, is a useful technique for cancer chemotherapy.

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

1. Introduction

Injectable polymers have drawn considerable attention as promising biomaterial for drug delivery and regenerative medicine. Multiple biocompatible and biodegradable polymers are routinely employed as carriers of injectable DDS in order to diminish the drug side-effect, especially for local administration and delivery when used for anticancer chemotherapy. Many polymeric materials for injectable DDS, such as nanoparticle [1–3], microsphere [4,5], polymeric micelles [6–8], liposomes [9–12], and hydrogel system [13,14], have been investigated and developed. Although some formations of them have succeeded in clinical applications there still remain many problems that need to be addressed. One of the recent manifestations of stimuli-responsive polymers lies in in-situ forming DDS by sol–gel transition for in-situ forming hydrogel because it is feasible to use them as carrier for local administration [15]. As representatives of stimuli-responsive polymer for in-situ forming hydrogel, there are several candidates that include thermoresponsive polymers such as *N*-isopropyl acrylamide copolymer [16], polyethylene glycol–polypropylene glycol–polyethylene glycol (PEG–PPG–PEG) triblock copolymer [17], and polyethylene glycol–poly L-lactic acid–polyethylene glycol (PEG–PLLA–PEG) triblock copolymer [18]. These thermoresponsive polymers exhibit thermo-dependent sol–gel transition in aqueous solution via hydrophobic interaction. The advantage of these kinds of polymers is in their ability to avoid toxic cross-linkers which are usually employed to form hydrogel. However, local injection of thermoresponsive polymers is

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doi:10.1016/j.ejpb.2007.03.020

operationally difficult. Their thermoresponsiveness is too sensitive for injection by syringe pump and for this reason they must be cooled down to below the transition temperature before they can be injected. Furthermore, ion-mediated cross-linked hydrogels, such as alginates, which form a gel upon contact with divalent cations, have been widely researched as injectable in-situ forming DDS and tissue engineering because of their biocompatibility [19,20]. Many alginate derivatives such as lectin-modified alginate [21] and RGD containing alginate [22] have also been synthesized. Despite many of their applications, alginate hydrogels have limited use because of their low shelf lives.

Recently, we have developed a novel tissue adhesive consisting of biomacromolecules such as collagen, gelatin and human serum albumin, and organic acid derivatives with active ester groups [23–27]. The bonding strength and biocompatibility of these adhesives to soft tissues were found to be superior to the ones obtained from the commercially available surgical glues such as cyanoacrylate derivatives [28,29], fibrin glue [30] and biomolecules-aldehydes glue [31]. The ability of an adhesive to bind to living tissue is very important in order to immobilize the tissue at some local site after administer drugs into a subject's body. The novel tissue adhesive, therefore, has high potential for use as injectable in-situ forming DDS (Fig. 1). The work on injectable in-situ forming DDS using fibrin glue for cancer chemotherapy has already been reported, however, desirable results were not achieved during the long-term release of carcinostatics [32–34]. In an earlier work, we prepared an injectable in-situ forming DDS that was composed of human serum albumin (HSA) and tartaric acid derivative (TAD) (Fig. 2). And there it was also shown that physicochemical properties such as gelation time, gel strength and bonding strength of HSA–TAD gel could be controlled with material composition [35]. Furthermore, we demonstrated long-term release of doxorubicin hydrochloride (DOX) from HSA–TAD gel for approximately up to 100 h in the in vitro.

Their antitumor effect was evaluated using human colon carcinoma (WiDr) that was implanted subcutaneously onto immunodeficient mouse. Tumor volume and blood plasma level of DOX were then evaluated.

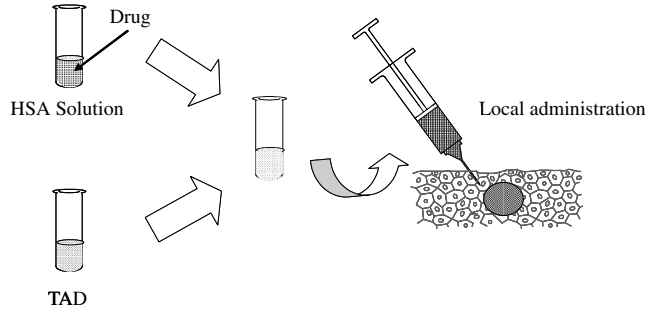

Fig. 1. Injectable in-situ forming drug delivery system with HSA–TAD adhesive.

2. Materials

Human serum albumin was obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). Tartaric acid, *N*-hydroxysuccinimide (HOSu) and doxorubicin hydrochloride (DOX) 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 HPLC or analytical grade without further purification.

3. Methods

3.1. Synthesis of TAD

TAD was prepared by a similar procedure 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 with rotary evaporation under a reduced pressure to remove THF. The resulting mixture was recrystallized to yield pure TAD. The resulting TAD was confirmed by ^{1}H NMR spectroscopy and elemental analysis, as well as by a previous work [35].

3.2. Determination of gelation time

HSA was dissolved in PBS (0.1 M, pH 7.4) at various concentrations. HSA solutions were weighed for 0.5 g in a polypropylene tube, and then different amounts of TAD (0.05, 0.075, and 0.1 mmol/0.8 g of HSA sol) were added to HSA solution and stirred vigorously. In the case of the DOX containing HSA–TAD, the DOX was added to HSA solution before adding TAD.

3.3. Measurement of bonding strength

Bonding strength of HSA–TAD with/without DOX was measured by using collagen casing which adhered on test pieces (10×10 mm) at the one end of a PET film (10×40 mm). Seventy microliters of HSA–TAD with/without DOX was first applied to collagen casing. The other test piece was then placed on the first layer. The bonding area was set at 10×10 mm. After 10 min at 37 °C, the bonding strength, as shearing bonding strength, was measured by tensile testing machine (TA-XT2i, Eko Instruments Co., Ltd., Tokyo, Japan). Three samples were tested to measure the same bonding strength ($n = 3$).

3.4. Determination of DOX

Determination of DOX was carried out by RF-HPLC. The HPLC system consisting of HPLC pump (PU-2080 plus), intelligent autosampler (AS-2055 plus), fluorescence detector (FP-2020 plus), column ovens (CO-2060) and degasser (DG-2080-53) was purchased from Jasco Inc. (Tokyo, Japan). The separation was carried out using reverse-phase

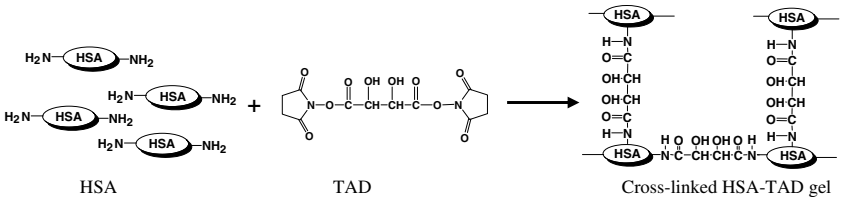

Fig. 2. Cross-linking reaction of HSA by TAD.

C_{18} column (Cosmosil, Nacalai Tesque, Inc., Japan). Mobile phase was prepared as mixed solvent (water/acetonitrile/acetic acid = 77:22:1 (v/v/v)) 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.

3.5. 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 (∅10.0 × 5.0 mm) were prepared using a mold made of silicone rubber spacer and two glass plates. These gels were then immersed into 100 ml of 0.1 M PBS (pH 7.4) at 37 °C. One-half milliliter of the release medium was corrected periodically and replenished with 0.5 ml of fresh PBS. The amount of DOX in the release medium was determined by RF-HPLC.

3.6. Cell line and culture conditions

A human colon carcinoma (WiDr) was purchased from Health Science Research Resources Bank (Osaka, Japan). WiDr cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich Co., St. Louis, MO, USA) supplemented with 10 v/v% fetal bovine serum (JRH Bioscience, Inc., USA) and 0.5 v/v% penicillin–streptomycin (Gibco, USA). The cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

3.7. Evaluation of antitumor effect

The suspension of WiDr cells (1.0×10^7 cells/0.2 ml of physiological saline) was implanted subcutaneously onto immunodeficient mouse (BALB/c AnNCrj – un) (Charles River Laboratories Japan, Inc., Japan). The in vivo experiments were started when the tumor grew up to a diameter of approximately 1.0 cm. Aqueous solution of DOX (DOX 0.25 mg/0.2 ml) and HSA–TAD (33 w/w% 0.1 mmol/0.8 g of HSA sol) with/without DOX was injected around the tumor by syringe pump with 24 G hypodermic needle. Volume of injection and dosage of DOX for a mouse were set at 0.2 ml and 0.25 mg, respectively. Afterward, measurement of the blood plasma level of DOX, body weight and tumor volume was periodically carried out. The blood plasma level of DOX was measured by RF-HPLC after pretreatment as follows. Four times volume of acetonitrile (400 μl) was added to blood plasma (100 μl) and the mixture was then vigorously stirred. The precipitate was removed with centrifuging and syringe filter (DISMIC-13NP 0.45 μm, Advantec, Ltd., Japan), then the concentration of DOX in the solution was measured. The short diameter (D_s) and long diameter (D_l) were periodically measured and the tumor volume was calculated using the following equation [36];

$$\text{Tumor volume} = D_s^2 \times D_l/2$$

4. Results and discussion

4.1. Characterization of TAD

The synthesis of TAD was carried out by the standard protocol using HOSu and DCC [37]. Characterization of TAD was performed by ^{1}H NMR and elemental analysis. The result showed that highly purified TAD could be obtained with a satisfactory yield of approximately 60%.

4.2. Physicochemical properties of HSA–TAD containing DOX

In our previous report, we had indicated that physicochemical properties of HSA–TAD could be controlled with material composition. Furthermore, it was demonstrated that gelation time of HSA–TAD could be changed by the addition of DOX (0.25 mg/0.5 g of HSA–TAD), although final cross-linking density was not influenced by it. As shown in Fig. 3, the gelation time of HSA–TAD with/without DOX depended on the change in HSA concentration. The concentration of HSA was varied from 30 to 35 w/w%. Gelation time of HSA–TAD (HSA – 30 w/w%) was approximately 116 s which was then shortened with an increasing HSA concentration. Furthermore, the gelation time of HSA–TAD was lengthened by the addition of DOX. This behavior was more remarkable than the one mentioned in the previous report. At 30 w/w% of HSA, the gelation time was lengthened approximately by 35 s by the addition of DOX. This behavior suggested that amino group in DOX reacted with active ester groups of TAD and the pH value of HSA. It was also suggested that TAD mixture decreased with free-HOSu during cross-linking reaction. These results indicated that the gelation time can be conveniently controlled by material composition.

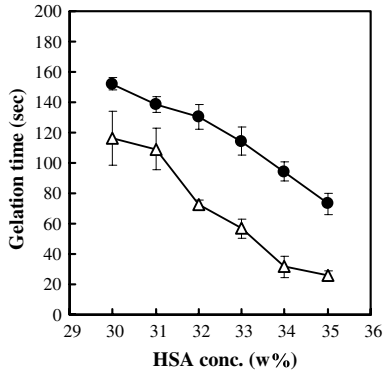

Fig. 3. Dependence of HSA concentration on gelation time of HSA–TAD with (●) and without (△) DOX. Error bars exhibit standard deviations ($n = 3$).

As shown in Fig. 4, the bonding strength of HSA–TAD with/without DOX was measured as tensile-shearing adhesive strength using the test pieces prepared with collagen casing. The bonding strength of HSA–TAD without DOX gradually increased with an increasing HSA concentration, that is, it increased from 93.0 ± 2.2 to 171.7 ± 9.7 with changes in HSA concentration from 30.0 to 35.0 w/w%. DOX containing HSA–TAD also indicated the increase in the bonding strength with HSA concentration. Change of bonding strength corresponded to the cross-linking density of HSA–TAD gel, that is, the cross-linking density increased with an increasing HSA concentration. Furthermore, the addition of DOX did not influence as well as the previous report. These results suggested that DOX does not affect the final cross-linking density of HSA–TAD gels, and indicated that HSA–TAD with DOX has good bonding strength for use as injectable in-situ forming DDS.

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

We have already demonstrated the long-term release of DOX from HSA–TAD gel for approximately up to 100 h even though DOX is very unstable in release medium (0.1 M PBS, pH 7.4) [35]. The in vitro release profile of highly concentrated DOX containing HSA–TAD gels is indicated in Fig. 5. The amount of DOX released from HSA–TAD gels was found to decrease with an increase in the cross-linking density of HSA–TAD gels. Furthermore, the release profile of DOX from all gels reached a maximum after the first 24 h. The release of DOX then began to decrease and the trend continued for approximately up to 100 h. These results suggested that DOX in HSA–TAD gel is more stable than in external solution, because pH value in HSA–TAD gel is lower than that of external solution caused by the formation of free-HOSu after cross-linking reaction. Although the toxicity of free-HOSu is concerned, it was reported that HOSu has no mutagenic activity [38]. Therefore, highly concentrated DOX containing HSA–TAD indicated appropriate characteristics for in-situ forming DDS.

4.4. Antitumor effect of DOX containing HSA–TAD

In order to use DOX containing HSA–TAD for the in-situ forming DDS, appropriate characteristics have to be optimized with material composition. Therefore, DOX containing HSA–TAD was first injected into an immunodeficient mouse that was implanted with human colon carcinoma (WiDr). HSA and TAD concentration was fixed at 33 w/w% and 0.1 mmol/0.8 g of HSA sol, respectively, because this composition was known to have the appropriate gelation time, good bonding strength and desirable release profile of DOX. Four conditions were evaluated using mice without procedure (control), DOX solution (free-DOX), injection of HSA–TAD without DOX (DOX (−)), and DOX containing HSA–TAD (DOX (+)). As shown in Fig. 6, in the case of HSA–TAD (DOX (+)) group, the change in the blood plasma level of DOX could be detected only after 72 h. On the other hand, DOX was detected in the blood plasma in free-DOX group just after 6 h. No DOX was detected in control and HSA–TAD (DOX (−)) groups. In the in vitro release test, HSA–

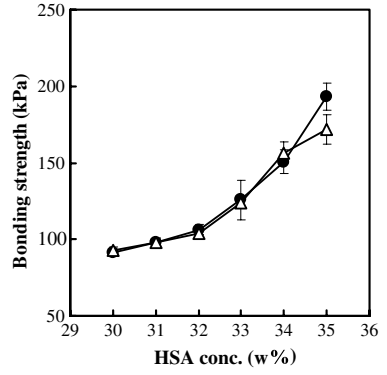

Fig. 4. Dependence of HSA concentration on bonding strength of HSA–TAD with (●) and without (△) DOX. Error bars exhibit standard deviations ($n = 3$).

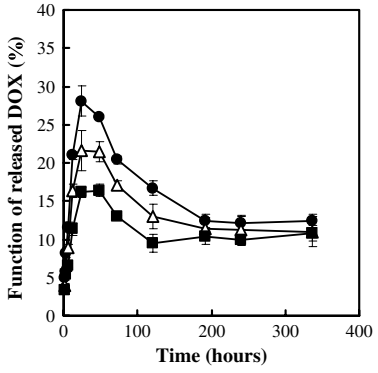

Fig. 5. In vitro release profiles of DOX from HSA–TAD gels consisting of 30 w/w% HSA (●), 33 w/w% HSA (△), 35 w/w% HSA (■) with 0.1 mmol/0.8 g of HSA sol. Error bars exhibit standard deviations ($n = 3$).

TAD (DOX (+)) group showed long-term release of DOX for approximately up to 100 h. This result almost corresponded to that of the in vivo experiment. The blood plasma level of DOX was very low in free-DOX and HSA–TAD (DOX (+)) groups because DOX is very unstable in the blood.

Fig. 7 shows body weight change of WiDr cell implanted mice after the application of DDS. The body weight gradually decreased in all groups because of the growth of tumor. This result suggested that contribution by injection of HSA–TAD was negligible.

Change of tumor volume was also evaluated. As shown in Fig. 8, tumor volumes gradually increased in the cases of control, free-DOX, and HSA–TAD (DOX (−)) groups. On the other hand, decrease in tumor volume occurred during the first 12 h after the injection of HSA–TAD (DOX (+)) group. When we injected HSA–TAD (DOX (+)) into the tumor, the tumor volume reduced to a minimum after 12 h, and then began to increase gradually. This tumor size of HSA–TAD (DOX (+)) group was smaller than that of the other groups. Because of the bonding ability of HSA–TAD (DOX (+)) to solid tumor for a long time, it is inferred that the local DOX concentration in solid tumor was maintained for long-term. This result indicates that HSA–TAD (DOX (+)) group had an excellent antitumor effect for use as in-situ forming DDS.

5. Conclusion

Using a novel tissue adhesive HSA–TAD, DOX releasable in-situ forming DDS was developed. It was shown that the physicochemical properties and the release profile of DOX from HSA–TAD gel could be controlled with matrix composition. The release of DOX from HSA–TAD in the in vitro and the in vivo experiments was maintained for approximately up to 100 and 72 h, respectively. Furthermore, tumor volume was found to decrease by injection of DOX containing HSA–TAD. These results demonstrated that this novel tissue adhesive can be applied to the injectable in-situ forming DDS for cancer chemotherapy.

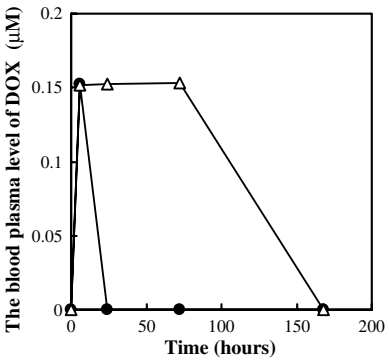

Fig. 6. Change in blood plasma level of DOX free-DOX (●), HSA–TAD (DOX (+)) (△) groups ($n = 3$).

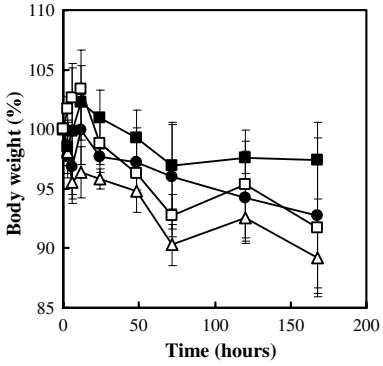

Fig. 7. Change in body weight of mice implanted WiDr cell in control (●), free-DOX (△), HSA–TAD (DOX (−)) (■) and HSA–TAD (DOX (+)) (□) groups. Error bars exhibit standard deviations ($n = 3$).

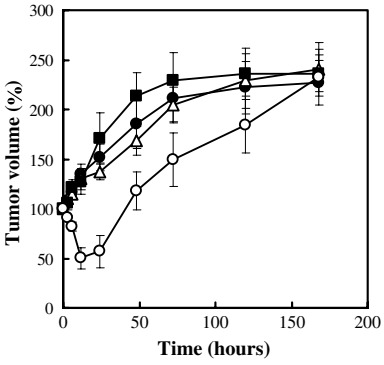

Fig. 8. Change in tumor volume of mice implanted with WiDr cell in control (●), free-DOX (△), HSA–TAD (DOX (−)) (■) and HSA–TAD (DOX (+)) (○) groups. Error bars exhibit standard deviations ($n = 3$).

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