In Situ Gellable Oxidized Citrus Pectin for Localized Delivery of Anticancer Drugs and Prevention of Homotypic Cancer Cell Aggregation

ARTICLE *in* BIOMACROMOLECULES · OCTOBER 2010

Impact Factor: 5.75 · DOI: 10.1021/bm1010068 · Source: PubMed

4 AUTHORS, INCLUDING:

Takayuki Takei
Kagoshima University
58 PUBLICATIONS **415** CITATIONS

In Situ Gellable Oxidized Citrus Pectin for Localized Delivery of Anticancer Drugs and Prevention of Homotypic Cancer Cell Aggregation

Takayuki Takei,* Mitsunobu Sato, Hiroyuki Ijima, and Koei Kawakami

Department of Chemical Engineering, Graduate School of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0385, Japan

Received August 27, 2010; Revised Manuscript Received October 4, 2010

The aim of this study was to develop in situ gellable hydrogels composed of periodate oxidized citrus pectin (OP) for localized anticancer drug delivery and evaluate the potential of OP to inhibit cancer metastasis. Doxorubicin (Dox) was coupled to OP by imine bonds. Adipic dihydrazide (ADH) was used for cross-linking of the Dox-OP conjugates. The Dox-OP conjugate solution gelled within 2 min after addition of ADH. The release rate of Dox from the hydrogels was controllable by an additive amount of ADH. The released Dox retained anticancer activity. OP was shown to have a potency to prevent homotypic cancer cell aggregation compared to unmodified citrus pectin, strongly suggesting that OP released from hydrogels in vivo will inhibit cancer metastasis. These results indicate that OP hydrogels have the potential to prevent progression of primary cancer by the released Dox and generation of metastatic cancer by the released OP.

Introduction

A common approach to the treatment of cancer is the intravenous administration of anticancer drugs. However, to achieve complete eradication of cancers, the drugs must be administered in high doses, leading to adverse side effects such as severe immunosuppression, myelosuppression, nephrotoxicity, and cardiotoxicity.[1−3] Localized delivery of drugs incorporated in biodegradable hydrogels can improve both the safety and efficacy of systemic chemotherapy by achieving controlled release of the drugs.[1,4,5] In particular, localized delivery using an in situ gellable hydrogel has attracted much attention because this type of hydrogel has the advantage over preformed hydrogels that it does not require surgical procedures for implantation.[6]

To date, various types of in situ gellable hydrogels with biodegradability have been reported.[7−11] Among these, hydrogels composed of polysaccharide oxidized by periodate (dextran, hyaluronic acid, chondroitin sulfate, carboxymethylcellulose, gum arabic, and konjac glucomannan) have been widely investigated.[12−16] Periodate oxidation leads to the cleavage of diol groups in the monosaccharide units of polysaccharides, resulting in the formation of aldehyde groups in the polymer backbones.[17] The aldehyde groups can be utilized both for immobilization of a drug possessing amino groups to the polymer backbones and for hydrogel formation with cross-linkers possessing amino groups or hydrazide groups via the formation of imine or hydrazone bonds.[18−20] Periodate oxidation also results in degradation of polysaccharides due to the cleavage of internal glycosidic linkages.[17,21,22] Furthermore, the cleavage of diol groups in monosaccharide units by periodate enhances the biodegradability of the polysaccharides.[15,20,23] These features of periodate oxidation are very attractive for utilization of polysaccharides of nonhuman origin whose degrading enzymes are not synthesized by humans, such as alginate, because lowering the molecular weight and enhancing biodegradability of the polysaccharide by periodate oxidation facilitates elimination of the polymer from the body through the kidney.[15,20,23]

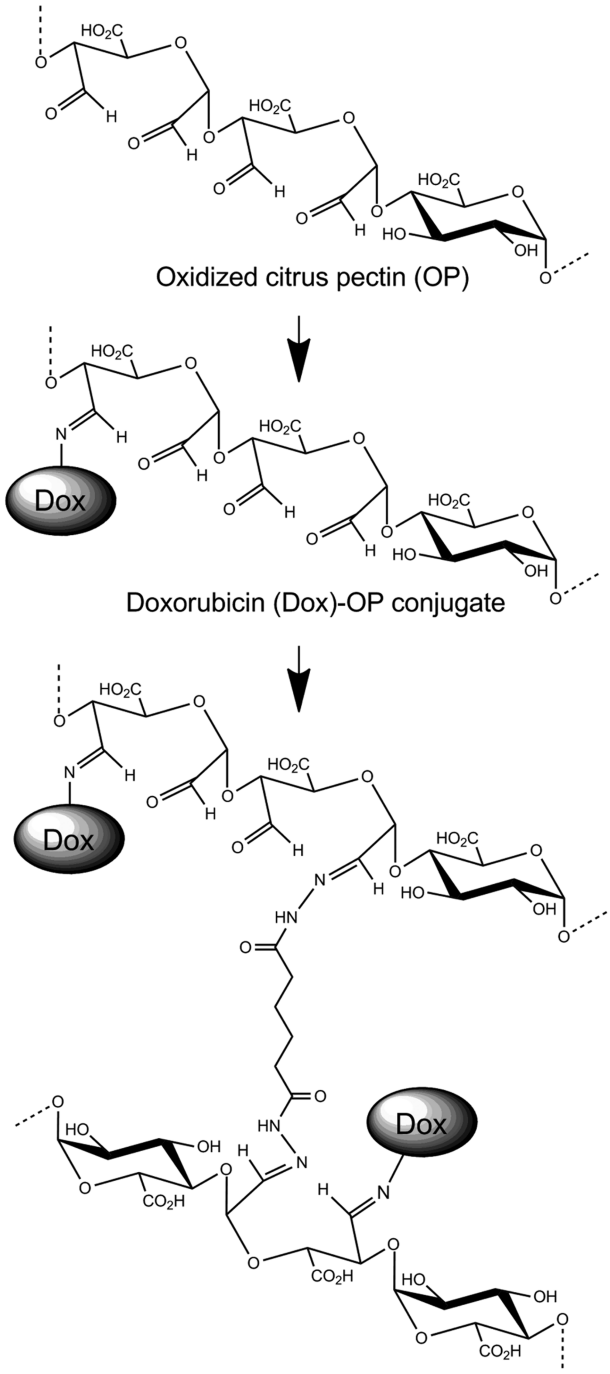
The purpose of the present study was to develop in situ gellable hydrogels composed of periodate oxidized citrus pectin (OP) for cancer chemotherapy. To the best of our knowledge, there have been no reports in which OP was utilized as a base material for in situ gellable hydrogels. Pursuit of new hydrogel materials is essential for development of an academic field concerning anticancer drug delivery because previously reported hydrogel materials do not satisfy all requirements for anticancer drug delivery, such as high biocompatibility, suitable biodegradability, and release rate of the drug. Citrus pectin (CP) is a branched, complex polysaccharide extracted from citrus fruits. Primarily, it contains large amounts of 1,4-linked α-D-galacturonic acid and its methyl ester along with natural sugars such as galactose, glucose, and mannose.[24,25] Currently, CP is attracting significant attention as a possible biomedical material because of its high biocompatibility.[24,26−28] An obstacle to utilizing CP as a biomaterial is that implanted CP remains in the human body over a long term due to the lack of the a degrading enzyme of the polymer, which might lead to unfavorable biological responses. However, considering that periodate oxidation of CP would result in lowering the molecular weight and enhancing biodegradability of the polymers, it is expected that OP can be easily eliminated from the body. In this study, an OP-based hydrogel incorporating doxorubicin (Dox), an anticancer drug, was prepared by cross-linking Dox-OP conjugates with adipic dihydrazide (Scheme 1). The primary aim of this study was to evaluate the characteristics of the OP hydrogels: (i) gelling behavior, (ii) degradation rate, (iii) Dox release rate, and (iv) anticancer activity of Dox released from the hydrogels.

There is a possibility that OP itself has potency in preventing the generation of metastatic cancers. A pH-modified citrus pectin (MCP), which has a smaller molecular weight than unmodified CP, has been reported to have positive effects for cancer therapy, including prevention of cancer metastasis and inducement of apoptosis in neoplastic cells.[25,29] It is expected that OP, which

* To whom correspondence should be addressed. Tel. and Fax: +81-92-802-2768. E-mail: takei@chem-eng.kyushu-u.ac.jp.

10.1021/bm1010068
Published on Web 10/28/2010

Scheme 1. Preparation of Dox-Loaded OP Hydrogel



would also have a lower molecular weight than CP, released from the biodegradable OP hydrogel would have an identical potency to MCP. This increases the availability of OP hydrogels in cancer therapy. Thus, in addition to the primary aims, we evaluated the effect of OP on inhibiting homotypic cancer cell aggregation, to obtain experimental knowledge about the potential positive effect of the OP hydrogels for preventing cancer metastasis.

Experimental Section

Materials and Cells. USP grade GENU citrus pectin (CP, content of galacturonic acid: ≥74%, degree of esterification: 74%) was kindly donated by SANSHO Co. Ltd. (Osaka, Japan). Doxorubicin hydrochloride (Dox), sodium periodate, and adipic dihydrazide (ADH) were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Dox dissolved in dimethylsulfoxide at 25 mg/mL was used in all experiments. A human liver carcinoma cell line (HepG2) and mouse melanoma cell line (B16-F1) were purchased from Riken Bioresource Center Cell Bank (Tsukuba, Japan).

Synthesis of Oxidized Citrus Pectin (OP). Periodate oxidation of CP was conducted as previously reported.[1] Briefly, sodium periodate aqueous solution was added to CP aqueous solution and stirred at room temperature in the dark for 6 h. The final concentration of CP in the mixture was 2% (w/v). The concentration of periodate was varied to obtain OP with different degrees of oxidation. The resultant polymer solution was dialyzed against deionized water using a dialysis membrane (MWCO: 14000) for 3 d with several changes of the water. The removal of periodate was ascertained by mixing the final dialysate with the same volume of 1% (w/v) silver nitrate solution and confirming the absence of precipitate. The resultant polymer was lyophilized to obtain OP powder.

The degree of oxidation of OP was determined using the hydroxylamine hydrochloride titration assay.[30] Briefly, 0.1 g of OP was dissolved in 25 mL of 0.25 M hydroxylamine hydrochloride−methyl orange solution for 3 h and then titrated with 0.1 M sodium hydroxide solution.

Viscosities of 0.5% (w/v) OP and CP aqueous solutions at room temperature were measured using a Brookfield Viscometer (model DV-E, MA).

The contents of galactose in CP and OP were determined as follows. CP or OP were added to 72% (w/w) sulfuric acid aqueous solution and stirred at room temperature for an hour. Deionized water was added to the solution to lower the concentration of sulfuric acid (4% (w/w)) and then the solution was autoclaved at 121 °C for an hour. After the resultant solution was neutralized with sodium hydroxide, the content of galactose monomer was measured using a high-performance liquid chromatography (HPLC) system (LC-10ADVP, Shimadzu, Kyoto, Japan) equipped with a TSKgel Sugar AXI column. Postcolumn reaction with arginine was adopted for the sensitive determination of galactose.[31] The mobile phase was 0.5 M borate buffer and the effluents were monitored fluorometrically at an excitation wavelength of 320 nm and an emission wavelength of 430 nm.

Preparation of OP Hydrogels and Dox-Loaded OP Hydrogels. OP and ADH were dissolved in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (CMF-PBS, pH 7.4), respectively. An OP solution (0.4 mL) was mixed with ADH solution (0.1 mL) in a vial and then incubated at 37 °C for gelation of the mixture. The concentration of OP in the resultant mixture was fixed at 3.0 or 5.0% (w/v). The molar ratio of hydrazide groups on ADH to aldehyde groups on OP (ADH/OP ratio) in the mixture were varied (1.00, 0.50, 0.33, 0.20, 0.10, and 0.03) using ADH solutions of different concentrations (6−330 mM). Gelation was evaluated using the conventional inverted test tube method.[32] When we prepared Dox-loaded hydrogels, 20 μL of Dox solution (25 mg/mL) was first added to 0.4 mL OP solution and stirred at room temperature for 5 min to form the Dox-OP conjugate. Subsequently, the solution was mixed with 0.1 mL ADH solution and incubated at 37 °C. The concentration of Dox in the mixture was fixed at 1 mg/mL in all experiments.

Determination of Incorporated Dox. The amount of Dox incorporated into the OP hydrogel was determined according to a previously reported method.[1] Fluorescein sodium was used as a control. A 0.4 mL OP solution was mixed with 20 μL Dox solution (25 mg/mL) or 20 μL of fluorescein sodium solution (25 mg/mL) and stirred at room temperature for 5 min. Subsequently, the solutions were mixed with 0.1 mL of ADH solution and incubated at 37 °C for 5 min to form hydrogels. The concentrations of OP and ADH/OP in the hydrogels were 3.0% (w/v) and 1.0, respectively. The hydrogels were lyophilized and crushed to obtain powders. The powders were placed in a well-stirred CMF-PBS solution at room temperature for 10 min, and the suspension was filtered to remove fine particles. The concentration of Dox in the filtered CMF-PBS solution was spectrophotometrically

Table 1. Experimental Conditions for the In Vitro Assay for Inhibition of Homotypic Cancer Cell Aggregation[a]

condition	OP (% (w/v)) [aldehyde group (μmol/mL)]	CP (% (w/v))	Dox (μmol/mL)	lactose (μmol/mL)
control				
1	0.1 [2.7]			
2	0.1 [2.7]		5.8×10^{-2}	
3		0.1		
4		0.1		150.0

[a] All conditions contained ADH at 27.0 μmol/mL (hydrazide group: 54.0 μmol/mL).

determined at a wavelength of 483.5 nm in comparison to an established standard curve. The concentration of fluorescein sodium was fluorometrically determined at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Volume Change of OP Hydrogels. Rectangular OP hydrogels (0.5 mL) without Dox were prepared by pouring a mixture of OP and ADH solutions into a mold and incubating at 37 °C for 15 min. The concentrations of OP in the hydrogels were fixed at 3 and 5% (w/v). After removal from the mold, the hydrogels were added to 10 mL of CMF-PBS and gently shaken at 37 °C. CMF-PBS was exchange with fresh buffer every 3 d. At regular intervals, volume of the hydrogel was determined based on its wet weight (we hypothesized that a volume of OP hydrogel equaled to its weight because the hydrogel was mainly composed of water). The ratio of a volume of hydrogel at $t = t$ (W_t) to that at $t = 0$ (W_0) was calculated by the following equation.

$$\text{volume ratio} = W_t \times 100/W_0$$

Dox Release Behavior. Rectangular Dox-loaded hydrogels were prepared as follows. An OP solution (0.4 mL) was mixed with 20 μL of Dox solution (25 mg/mL) in a mold at room temperature for 5 min. The solution was then mixed with 0.1 mL of ADH solution and incubated at room temperature for 15 min. The concentration of OP in the hydrogels was fixed at 3% (w/v). After removal from the mold, the hydrogels were immersed in 2.5 mL of CMF-PBS and gently shaken at 37 °C. At appropriate intervals, whole aliquots were replaced with fresh CMF-PBS (2.5 mL). The amounts of Dox in the samples were spectrophotometrically determined at a wavelength of 483.5 nm. The samples were kept at −80 °C until an in vitro anticancer activity assay was carried out.

In Vitro Anticancer Activity. Anticancer effects of Dox released from OP hydrogels were determined by the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8) assay in HepG2 cells.[33] Samples collected as described in Dox Release Behavior and pure Dox solution (25 mg/mL) were diluted with CMF-PBS. HepG2 cells were cultivated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C. The cells were seeded on a 96-well culture dish at a cell density of 2×10^4 cell/well (180 μL medium per well). After 24 h of cultivation at 37 °C, the diluted samples or pure Dox solution (20 μL) were added to each well. After 48 h of cultivation at 37 °C, aliquots were removed and the cells were washed twice with cell culture medium. Subsequently, culture media containing 10% (v/v) WST-8 reagent were added to each well and incubated for 1.5 h at 37 °C. Absorbance of the medium at 450 nm was measured using a spectrophotometer. The values for 50% cytotoxicity (IC_{50}) of the released fractions and pure Dox were determined based on the absorbance.

In Vitro Assay for Inhibition of Homotypic Cancer Cell Aggregation. This assay was conducted as described by a previous report.[25] Test matter was dissolved in Ca^{2+}-free Krebs-Ringer HEPES-buffered solution (CF-KRH, pH 7.4), as shown in Table 1. For condition 2, OP solution was first mixed with Dox and then incubated for 30 min to form a Dox-OP conjugate. ADH was then added to the solution.

Table 2. Characteristics of Unmodified CP and OP

periodate equivalent[a]	degree of oxidation (%; $n = 3$)	viscosity (mPa·s)[b]	galactose content (g/100 g of pectin)
unmodified CP		7.68	5.8
0.25	24.8 ± 0.7	2.55[c]	4.1[c]
0.50	42.3 ± 2.5		
0.75	49.9 ± 1.4		
1.00	53.5 ± 4.4		

[a] Periodate equivalent means the molar ratio of sodium periodate to monosaccharide units of CP. [b] Viscosity of 1% (w/v) sodium hexametaphosphate aqueous solution (pH 4.5) with dissolved 0.5% (w/v) CP or OP. [c] The degree of oxidation of the OP used was 24.5%.

The condition 1 test solution was prepared without addition of Dox under an otherwise identical protocol to that used for condition 2. We confirmed that all test solutions did not gel. B16-F1 cells were suspended in each solution at 1×10^6 cells/mL. The suspension (0.6 mL) was placed in glass tubes and gently shaken at 4 °C for an hour. The number of single cells in each suspension was counted using a hemocytometer. The aggregation ratio of the cells was calculated using the following equation:

$$\text{aggregation ratio} = (1 - N_s/N_t) \times 100 \quad (1)$$

where N_s and N_t represent the single cell number after shaking and total cell number, respectively.

Results and Discussion

Synthesis of Oxidized Citrus Pectin (OP). The number of aldehyde groups on periodate oxidized polysaccharide molecules influences the characteristics of the oxidized polysaccharide-based hydrogel such as mechanical strength and the amount of incorporated Dox.[14] The number of aldehyde groups depends on the amount of periodate utilized in the oxidation reaction. Thus, we investigated the effect of the molar ratio of sodium periodate to monosaccharide units of CP on the oxidation degree of CP under the hypothesis that CP consisted only of galacturonic acid (74%) and galacturonic acid methyl ester units (26%).

Table 2 shows that an increase in the molar ratio resulted in an increase in the oxidation degree (the number of aldehyde groups), as reported by others.[18,34] However, the oxidation degree was not directly proportional to the ratio. This would result from the degradation of CP molecules via cleavage of internal glycosidic linkages due to random attack by hydroxyl radicals formed by the spontaneous decomposition of periodate.[17,21,22] The fact that the viscosity of the OP solution was lower than that of the CP solution (Table 2) supports the above consideration. In the following experiments, we used OP with a low degree of oxidation (approximately 25%) because it had been predicted that an increase in OP oxidation degree would hasten the formation of imine bonds with ADH, making the formation of a homogeneous hydrogel network difficult.[12]

Preparation of OP Hydrogels and Dox-Loaded OP Hydrogels. In the present study, we attempted to prepare Dox-loaded OP hydrogels by the following procedure (Scheme 1). OP-Dox conjugates are prepared by the formation of imine bonds between the aldehyde groups on OP and the amino groups on Dox. Subsequently, Dox-loaded hydrogels are prepared by the formation of hydrazone bonds between residual aldehyde groups on OP and hydrazide groups on ADH. In this experiment, we first investigated the amount of ADH required to form OP hydrogels before incorporating Dox into the hydrogels. An OP solution was mixed with ADH solution and incubated at 37 °C.

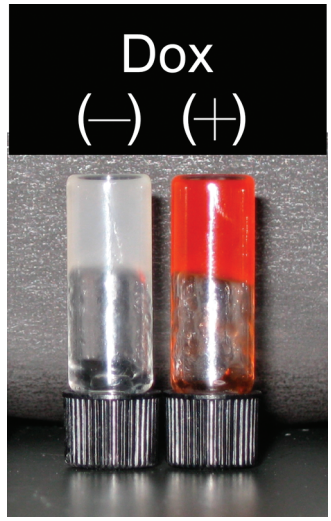


Figure 1. OP hydrogels with and without Dox at 2 min of incubation at 37 °C after addition of ADH solution (an ADH/OP ratio of 0.10).

The molar ratio of hydrazide groups on ADH to aldehyde groups on OP (ADH/OP ratio) in the mixtures containing 3% (w/v) OP was varied (1.00, 0.50, 0.33, 0.20, 0.10, and 0.03). Gelation of the mixtures was evaluated using the inverted tube test.[32] At an ADH/OP ratio of 0.03, the mixture did not gel even at 6 h after mixing the OP solution with the ADH solution. On the other hand, hydrogels were obtained within 2 min at ADH/OP ratios of 1.00, 0.50, 0.33, 0.20, and 0.10 (Figure 1). Similarly, in the case of an OP concentration of 5% (w/v), hydrogels were formed at ADH/OP ratios of 1.00, 0.50, 0.33, 0.20, 0.10, and not at 0.03. The reason that a mixture of ADH/OP ratio of 0.03 did not form a hydrogel would be that the degree of cross-linking was too low.

Based on the above results, we then confirmed whether OP solution containing Dox would gel by the addition of ADH at ADH/OP ratios of more than 0.10. Briefly, 0.4 mL OP solution was mixed with 20 μL Dox solution and incubated at room temperature for 5 min to couple Dox with OP. The solution was then mixed with 0.1 mL of ADH solution and incubated at 37 °C. The concentration of OP in the mixture after addition of ADH was fixed at 3.0% (w/v). Addition of Dox to OP solution would result in formation of imine bonds between the amino groups of the drug and the aldehyde groups on OP, indicating a decrease in the number of free aldehyde groups on OP. Therefore, we had to take into account the decreased number when we used a definition identity "ADH/OP ratio". However, even if all Dox could form conjugates with OP, the molar ratio of aldehyde groups on OP occupied by Dox was very small (2.2%). Therefore, we did not correct the value of ADH/OP ratio as a matter of convenience. At all ADH/OP ratios (1.00−0.10), hydrogels were formed within 2 min of adding ADH solution to an OP solution containing Dox (Figure 1). These results demonstrate that OP is promising as an in situ gellable material.

Subsequently, we confirmed that Dox was covalently coupled to OP in an incubation time of 5 min after mixing OP and Dox solutions. Dox-loaded hydrogels were prepared by almost the same procedure as described above (see Determination of Incorporated Dox). The concentrations of OP, ADH/OP ratio, and incubation time after mixing OP solution with Dox were 3.0% (w/v), 1.0, and 5 min, respectively. The hydrogels were lyophilized and crushed to form powders. The powders were placed in well-stirred CMF-PBS at room temperature for 10 min and the concentration of Dox released in the CMF-PBS, which was not covalently bound to OP, was spectrophotometrically determined. As a control, fluorescein sodium was used because it does not contain amine groups for coupling to OP and has a comparable molecular weight to Dox (the molecular weights of Dox and fluorescein sodium are 580 and 376 g/mol, respectively). Only less than 7% of the fluorescein sodium was retained in the hydrogel particles. On the other hand, 94.5 ± 0.3% of Dox remained in the particles. These results indicate that Dox was covalently coupled to the OP hydrogel.

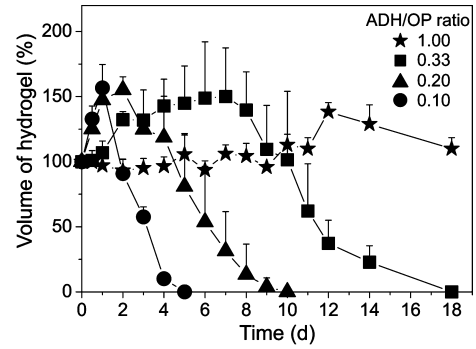


Figure 2. Degradation behavior of OP hydrogels containing 3% (w/v) OP. Bars: SD ($n = 3$).

Volume Change of OP Hydrogels and Dox Release Behavior. In this experiment, we first examined initial swelling ratio of OP hydrogels (the ratio of volume of the hydrogel at 1 day to that at 0 day) without Dox. The reason why we did not incorporate Dox into the hydrogels was because it had been predictable that there were not much difference in the swelling ratio between OP hydrogels with and without Dox due to quite-small number of aldehyde groups occupied by Dox in Dox-incorporating hydrogels as described above. Initial swelling ratios of the hydrogels of ADH/OP ratios of 1.00, 0.33, 0.20, and 0.10 (OP concentration: 3% (w/v)) were 97 ± 8, 107 ± 9, 147 ± 7 and 157 ± 18%, respectively (Figure 2). The increased initial swelling ratio with decreasing ADH/OP ratio would be due to a lower degree of cross-linking. The swelling ratios of OP hydrogels of an OP concentration of 5% were almost the same as those of OP concentration of 3% (w/v; data not shown). These results show that initial swelling of the hydrogels mainly depends on the ADH/OP ratio and not OP concentration, which corresponds to previous report.[1]

Subsequently, we investigated degradation rate of OP hydrogels based on their volume change. The concentration of OP in the hydrogels was fixed at 3.0% (w/v). It has been reported that hydrazone and imine bonds are labile to hydrolysis even at neutral pH,[1] which indicates that the hydrolysis of the hydrazone bonds in the polymer network contributes to degradation of OP hydrogels. Hydrogels at an ADH/OP ratio of 0.10 completely degraded within approximately 5 d (Figure 2). The degradation rate decreased with increasing ADH/OP ratio and the hydrogels with an ADH/OP ratio of 1.00 remained even after 18 d of incubation. It has been reported that hydrazide groups regenerated by hydrolysis of hydrazone bonds at the initial cross-linking sites allowed a recross-linking reaction of periodate oxidized polysaccharide molecules.[35] Thus, the reason that higher ADH/OP ratios resulted in slower degradation rates would be because increased amount of ADH facilitated the recross-linking reaction of OP molecules. The reason why the volume of the hydrogels increased before their decrease would be because hydrolysis of hydrazone bonds between OP and ADH resulted in creation of more space for retaining water in the polymer network.

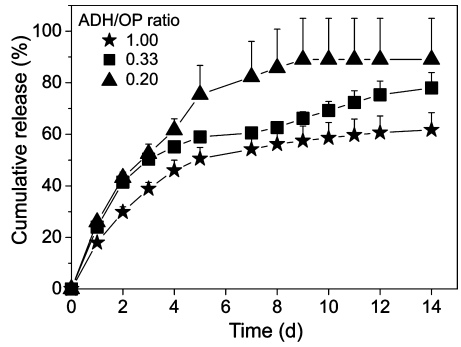


Figure 3. Release behavior of Dox from OP hydrogels containing 3% (w/v) OP. Bars: SD ($n = 3$).

Table 3. Inhibitory Activity of Dox Released from OP Hydrogels of ADH/OP Ratio of 0.20 Towards HepG2 Cells ($n = 3$)

sample	IC_{50} (μM)
pure Dox	0.17 ± 0.02
released Dox after 1 d	0.19 ± 0.03
released Dox after 3 d	0.28 ± 0.04
released Dox after 6 d	0.84 ± 0.16

Finally, we evaluated the effect of ADH/OP ratio on the release rate of Dox from OP hydrogels. The concentration of OP in the hydrogel was fixed at 3.0% (w/v). OP hydrogels of higher ADH/OP ratio showed slower Dox release rates (Figure 3). We confirmed that complete release of Dox was accompanied by the complete degradation of the hydrogel (ADH/OP ratio = 0.20). This indicates that (i) some of the free-Dox generated by hydrolysis of imine bonds was retained in the hydrogels by re-cross-linking with OP and (ii) Dox was released from the hydrogels not only as free drug but also as Dox-OP-ADH (also Dox-OP) conjugate. These results show that the release rate of Dox is controllable by the ADH/OP ratio.

In Vitro Anticancer Activity. The anticancer activity of Dox released from OP hydrogels of ADH/OP ratio of 0.20 was evaluated in vitro using a standard cytotoxicity assay with HepG2 cells.[36] Released drug was expected to contain free Dox, Dox-OP conjugate, and Dox-OP-ADH conjugate, as described above. The IC_{50} of pure Dox was 0.17 ± 0.02 μM (Table 3). The anticancer activity of Dox released after 24 h was almost identical to that of pure Dox, and the activity decreased with time. It was reported that Dox coupled with oxidized polysaccharide by imine bonds had lower anticancer activity than free Dox, which could subsequently be activated by the hydrolysis of the linkage between the oxidized polysaccharide and Dox.[37] This previous report indicates that free Dox was mainly released from the hydrogels in the first 24 h of incubation, and since then, Dox coupled to OP and OP-ADH (prodrug) was mainly released from the hydrogels. In addition, partial degradation of Dox would be one of the reasons that the activity of released Dox decreased with time.[1,38] We confirmed that neither aqueous OP nor ADH had a negative effect on the growth of HepG2 cells over the range of concentrations tested.

In Vitro Assay for Inhibition of Homotypic Cancer Cell Aggregation. Prevention of cancer metastasis is crucial in addition to regression of primary cancers for achieving the complete cure of cancers. Cancer metastasis includes the invasion of the cancer cells into surrounding tissues, dissemination through blood or lymph vessels, and growth at distant sites.[39] In the process of dissemination of cancer cells through blood and lymph vessels, the cells form homotypic cell aggregates to protect themselves from the hostile host environment.[40] Galectin-3, which is a type of lectin, one of a family of

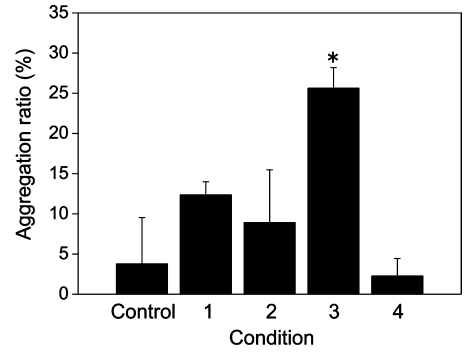


Figure 4. Aggregation ratio of B16-F1 cells incubated in each solution shown in Table 1. Bars: SD ($n = 3$). *$p < 0.05$ vs the other conditions.

carbohydrate-binding proteins, contributes to the formation of the cell aggregates. Galectin-3 has an affinity for β-galactoside moieties and is overexpressed on the cell surface of various cancer cells such as colon, head, neck, gastric, endometrial, thyroid, and breast carcinomas.[40−42] Formation of cancer cell aggregates is caused by binding of galectin-3 on the cells with galactose moieties of glycoproteins (also glycolipids) on the other cells.[39] Platt and Raz reported that unmodified CP promoted cancer cell aggregation and cancer metastasis.[25] On the other hand, they found that pH-modified citrus pectin (MCP) conversely prevented cancer metastasis by inhibiting formation of cancer cell aggregates.[25] MCP has a lower molecular weight than unmodified CP and nonbranched carbohydrate chains of essentially the same sugar composition as CP, which is prepared via degradation of the main galacturonic acid chain of CP under high pH conditions, followed by partial degradation of the natural carbohydrate chains under low pH condition.[25,40] The possible mechanisms for enhancement and prevention of the formation of cancer cell aggregates by CP and MCP are as follows. Branched CP with high molecular weight serves as cross-linker bridges among cancer cells via binding between galectin-3 on the cells and galactose moieties of CP, resulting in enhancement of cancer cell aggregation.[43] In contrast, MCP is unable to cross-link cancer cells due to its shorter carbohydrate chain. Alternatively, MCP covers individual cancer cells via binding between galectin-3 on the cells and galactose moieties of MCP, resulting in the prevention of cell aggregation. Considering that OP also has a lower molecular weight than CP and almost identical galactose content to CP (Table 2), it was expected that OP molecules released from OP hydrogel injected into the body would have the same potency as MCP. Thus, we evaluated the potency of OP for inhibiting cancer metastasis. Specifically, we examined the inhibitory effect of OP on the formation of homotypic cancer cell aggregates by an in vitro assay established by Platt and Raz.[25] It was reported that there was a good correlation between the propensity of cancer cells to undergo homotypic aggregation in the assay and their metastatic potential in vivo.[25] A mouse melanoma cell line (B16-F1), which overexpresses galectin-3 on the cell surface and has been used in the assay, was used as model cancer cells.

Detailed experimental conditions are given in Table 1. In view of the degradation mechanism of Dox-loaded hydrogel, we examined the inhibitory effects of both OP-ADH (condition 1) and Dox-OP-ADH conjugates (condition 2) on the formation of homotypic cancer cell aggregates. Excess of ADH was added to each solution to block the aldehyde groups of OP. The B16-F1 cells exhibited a low level of spontaneous homotypic aggregation (control, Figure 4), which corresponds to previous reports.[25] CP (condition 3) showed the highest aggregation ratio in all conditions. The presence of excess lactose (condition 4),

a inhibitor of galectin-3, resulted in inhibition of cell aggregate formation by CP, demonstrating that CP used in this study bound to the galectin-3 of B16-F1 cells.[25] Considering that OP has an almost identical galactose content to CP, these results indicate that OP also had high affinity to gelactin-3. OP-ADH and Dox-OP-ADH conjugates (conditions 1 and 2) showed much lower aggregation ratios than CP (condition 3) and the values were almost the same as that of the control condition. It was reported that MCP shows a much lower aggregation ratio than CP and the value was almost identical to the control condition without CP or MCP in this assay.[25] The previous report and the present results show that OP released from the hydrogel in vivo has excellent potential to inhibit the formation of homotypic cancer cell aggregates and subsequent cancer metastasis. This suggests that the present in situ gellable OP hydrogel is a promising material that enables both the prevention of progression of primary cancer by the released Dox and generation of metastatic cancer by the released OP.

Conclusions

In situ gellable hydrogels are promising vehicles for the localized delivery of anticancer drugs in a minimally invasive manner. In this study, we focused on OP as the in situ gellable material. Dox-loaded OP hydrogels were formed within 2 min at 37 °C by cross-linking Dox-OP conjugates with ADH. The release rate of Dox from the hydrogels could be controlled by the ADH/OP ratio. The released Dox retained anticancer activity. It was shown that OP had a potency to prevent homotypic cancer cell aggregation compared to unmodified CP, strongly suggesting that OP released from hydrogels in vivo will inhibit cancer metastasis. These results indicate that OP hydrogels have the potential to prevent both progression of primary cancer by the released Dox and generation of metastatic cancer by the released OP.

Acknowledgment. This research was partially supported by a grant from the Fukuoka Foundation for Sound Health.

References and Notes

(1) Bouhadir, K. H.; Kruger, G. M.; Lee, K. Y.; Mooney, D. J. *J. Pharm. Sci.* **2000**, *89*, 910–919.
(2) Lowenthal, R. M.; Eaton, K. *Hematol. Oncol. Clin. North. Am.* **1996**, *10*, 967–990.
(3) Kleinszanto, A. J. P. *Prog. Clin. Biol. Res.* **1992**, *374*, 167–174.
(4) Il Cho, Y.; Park, S.; Jeong, S. Y.; Yoo, H. S. *Eur. J. Pharm. Biopharm.* **2009**, *73*, 59–65.
(5) Chun, C.; Lee, S. M.; Kim, C. W.; Hong, K. Y.; Kim, S. Y.; Yang, H. K.; Song, S. C. *Biomaterials* **2009**, *30*, 4752–4762.
(6) Sakai, S.; Hirose, K.; Taguchi, K.; Ogushi, Y.; Kawakami, K. *Biomaterials* **2009**, *30*, 3371–3377.
(7) Kakinoki, S.; Taguchi, T. *Eur. J. Pharm. Biopharm.* **2007**, *67*, 676–681.
(8) Bhattarai, N.; Gunn, J.; Zhang, M. *Adv. Drug Delivery Rev.* **2010**, *62*, 83–99.
(9) Chen, T.; Embree, H. D.; Brown, E. M.; Taylor, M. M.; Payne, G. F. *Biomaterials* **2003**, *24*, 2831–2841.
(10) Bryant, S. J.; Davis-Arehart, K. A.; Luo, N.; Shoemaker, R. K.; Arthur, J. A.; Anseth, K. S. *Macromolecules* **2004**, *37*, 6726–6733.
(11) Yu, L.; Ding, J. D. *Chem. Soc. Rev.* **2008**, *37*, 1473–1481.
(12) Liu, Y.; Chan-Park, M. B. *Biomaterials* **2009**, *30*, 196–207.
(13) Ito, T.; Yeo, Y.; Highley, C. B.; Bellas, E.; Benitez, C. A.; Kohane, D. S. *Biomaterials* **2007**, *28*, 975–983.
(14) Dawlee, S.; Sugandhi, A.; Balakrishnan, B.; Labarre, D.; Jayakrishnan, A. *Biomacromolecules* **2005**, *6*, 2040–2048.
(15) Nishi, K. K.; Jayakrishnan, A. *Biomacromolecules* **2004**, *5*, 1489–1495.
(16) Yu, H. Q.; Lu, J.; Xiao, C. B. *Macromol. Biosci.* **2007**, *7*, 1100–1111.
(17) Vold, I. M. N.; Christensen, B. E. *Carbohydr. Res.* **2005**, *340*, 679–684.
(18) Hudson, S. P.; Langer, R.; Fink, G. R.; Kohane, D. S. *Biomaterials* **2010**, *31*, 1444–1452.
(19) Balakrishnan, B.; Jayakrishnan, A. *Biomaterials* **2005**, *26*, 3941–3951.
(20) Bouhadir, K. H.; Hausman, D. S.; Mooney, D. J. *Polymer* **1999**, *40*, 3575–3584.
(21) Symons, M. C. R. *J. Chem. Soc.* **1955**, 2794–2796.
(22) Scott, J. E.; Thomas, D. P. P. *Carbohydr. Res.* **1976**, *52*, 214–218.
(23) Lee, K. Y.; Mooney, D. J. *Chem. Rev.* **2001**, *101*, 1869–1879.
(24) Liu, L.; Fishman, M. L.; Kost, J.; Hicks, K. B. *Biomaterials* **2003**, *24*, 3333–3343.
(25) Platt, D.; Raz, A. *J. Natl. Cancer Inst.* **1992**, *84*, 438–442.
(26) Liu, L.; Won, Y. J.; Cooke, P. H.; Coffin, D. R.; Fishman, M. L.; Hicks, K. B.; Ma, P. X. *Biomaterials* **2004**, *25*, 3201–3210.
(27) Majzoob, S.; Atyabi, F.; Dorkoosh, F.; Kafedjiiski, K.; Loretz, B.; Bernkop-Schnurch, A. *J. Pharm. Pharmacol.* **2006**, *58*, 1601–1610.
(28) Katav, T.; Liu, L.; Traitel, T.; Goldbart, R.; Wolfson, M.; Kost, J. *J. Controlled Release* **2008**, *130*, 183–191.
(29) Chauhan, D.; Li, G.; Podar, K.; Hideshima, T.; Neri, P.; He, D.; Mitsiades, N.; Richardson, P.; Chang, Y.; Schindler, J.; Carver, B.; Anderson, K. C. *Cancer Res.* **2005**, *65*, 8350–8358.
(30) Zhao, H.; Heindel, N. D. *Pharm. Res.* **1991**, *8*, 400–402.
(31) Mikami, H.; Ishida, Y. *Bunseki Kagaku* **1983**, *32*, E207−E210.
(32) Ganji, F.; Abdekhodaie, M. J.; Ramazani, A. *J. Sol-Gel Sci. Technol.* **2007**, *42*, 47–53.
(33) Maeda, A.; Yano, T.; Itoh, Y.; Kakumori, M.; Kubota, T.; Egashira, N.; Oishi, R. *Atherosclerosis* **2010**, *208*, 112–118.
(34) Sanderso, C. J.; Wilson, D. V. *Immunology* **1971**, *20*, 1061–1065.
(35) Lee, K. Y.; Alsberg, E.; Mooney, D. J. *J. Biomed. Mater. Res.* **2001**, *56*, 228–233.
(36) Ozkan, A.; Fiskin, K. *Pol. J. Pharmacol.* **2004**, *56*, 435–444.
(37) Tevyashova, A. N.; Olsufyeva, E. N.; Preobrazhenskaya, M. N.; Klyosov, A. A.; Zomer, E.; Platt, D. *Russ. J. Bioorg. Chem.* **2007**, *33*, 139–145.
(38) Le Bot, M. A.; Riche, C.; Guedes, Y.; Kernaleguen, D.; Simon, S.; Begue, J. M.; Berthou, F. *Biomed. Chromatogr.* **1988**, *2*, 242–244.
(39) Liu, F. T.; Rabinovich, G. A. *Nat. Rev. Cancer* **2005**, *5*, 29–41.
(40) Nangia-Makker, P.; Conklin, J.; Hogan, V.; Raz, A. *Trends Mol. Med.* **2002**, *8*, 187–192.
(41) Lotan, R.; Ito, H.; Yasui, W.; Yokozaki, H.; Lotan, D.; Tahara, E. *Int. J. Cancer* **1994**, *56*, 474–480.
(42) Inohara, H.; Honjo, Y.; Yoshii, T.; Akahani, S.; Yoshida, J.; Hattori, K.; Okamoto, S.; Sawada, T.; Raz, A.; Kubo, T. *Cancer* **1999**, *85*, 2475–2484.
(43) Inohara, H.; Raz, A. *Glycoconjugate J.* **1994**, *11*, 527–532.

BM1010068