

Horseradish peroxidase-catalyzed formation of hydrogels from chitosan and poly(vinyl alcohol) derivatives both possessing phenolic hydroxyl groups



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ABSTRACT

Horseradish peroxidase-catalyzed cross-linking was applied to prepare hydrogels from aqueous solutions containing chitosan and poly(vinyl alcohol) derivatives both possessing phenolic hydroxyl groups (denoted as Ph-chitosan and Ph-PVA, respectively). Comparing the hydrogels prepared from the solution of 1.0% (w/v) Ph-chitosan and 3.0% (w/v) Ph-PVA and that of 3.0% (w/v) Ph-chitosan and 1.0% (w/v) Ph-PVA, the gelation time of the former hydrogel was 47 s, while was 10 s longer than that of the latter one. The breaking point for the former hydrogel under stretching (114% strain) was approximately twice larger than that for the latter one. The swelling ratio of the former hydrogel in saline was about half of the latter one. Fibroblastic cells did not adhere on the former hydrogel but adhered and spread on the latter one. The growth of *Escherichia coli* cells was fully suppressed on the latter hydrogel during 48 h cultivation.

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1. Introduction

Preparation of hydrogels by blending or conjugation of two or more different polymers has been widely studied because of the unique properties of the resultant hydrogels. Hydrogels composed of both chitosan and poly(vinyl alcohol) (PVA) are one such material that shows unique properties that cannot be obtained using either of the components alone (Islam, Yasin, Bano, & Riaz, 2012; Koyano, Minoura, Nagura, & Kobayashi, 1998; Kweon & Kang, 1999; Liang, Liu, Huang, & Yam, 2009; Zhao et al., 2003). For example, Koyano et al. (1998) reported that adhesion and growth of fibroblast cells on chitosan/PVA composite hydrogels can be controlled by altering the ratio of chitosan and PVA. PVA is a water-soluble polymer that has excellent chemical resistance and physical properties. High biocompatibility and durability are attractive characteristics of PVA and PVA-based hydrogels when applying the materials to the biomedical field (Suci, Iwatsubo, Matsuda, & Nishino, 2004). Chitosan is a cationic polysaccharide, which is produced industrially by deacetylation of chitin and used widely in the food industry. At present, interest is growing in the use of chitosan in the medical

and pharmaceutical fields because of its high biocompatibility, biodegradability, and antimicrobial property (George & Abraham, 2006; Muzzarelli, 2009; Muzzarelli & Muzzarelli, 2009; Peniche, Arguelles-Monal, Peniche, & Acosta, 2003). For the conjugation of chitosan and PVA, a variety of methods have been reported such as irradiation by electron beam (Zhao et al., 2003) or ultraviolet light (Sabaa, Mohamed, Eltaweel, & Seoudi, 2012) as well as chemical cross-linking with glutaraldehyde (Abdeen, 2011; Costa, Barbosa-Stancioli, Mansur, Vasconcelos, & Mansur, 2009). In this study, we applied horseradish peroxidase (HRP)-catalyzed cross-linking to obtain hydrogels from derivatives of chitosan and PVA (Fig. 1). To achieve cross-linking by HRP, chitosan and PVA derivatives possessing phenolic hydroxyl (Ph) groups, denominated as Ph-chitosan and Ph-PVA, respectively, were used. Cross-linking of molecules by enzymes including HRP has also attracted increasing attention as a possible environmentally friendly route for polymerization of molecules (Gross, Kumar, & Kalra, 2001; Kobayashi, Uyama, & Kimura, 2001; Muzzarelli, Ilari, Xia, Pinotti, & Tomasetti, 1994; Rocasalbas et al., 2013). Because of the mild reaction conditions of enzymes, such cross-linking systems are applicable to encapsulation of biomolecules and even living cells in hydrogels for applications in pharmaceutical and biomedical fields (Ito et al., 2003; Lee, Chung, & Kurisawa, 2009; Sakai, Hashimoto, Ogushi, & Kawakami, 2007). HRP is a heme protein that catalyzes the

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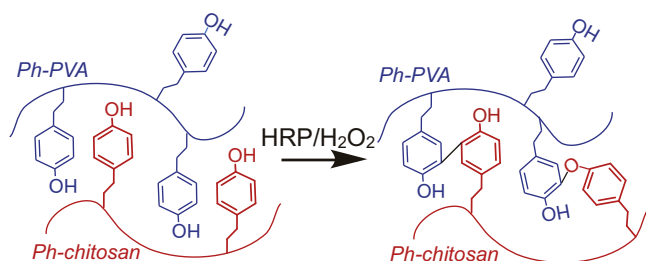


Fig. 1. Scheme for the formation of Ph-chitosan/Ph-PVA.

conjugation of phenol and aniline derivatives in the presence of H_2O_2 (Kobayashi et al., 2001). The major advantage of HRP, compared with other enzymes reported to be effective for obtaining hydrogels such as transglutaminase and tyrosinase, is more rapid hydrogelation (Teixeira, Feijen, van Blitterswijk, Dijkstra, & Karperien, 2012).

2. Materials and methods

2.1. Materials

A PVA derivative possessing carboxyl groups (PVA-COOH; AF-17, degree of hydrolysis: >96.5 mol%, viscosity of 4% solution: 30 mPa s) and chitosan (Chitosan LL, deacetylation: 0.80, MW: 50–100 kDa) were kindly donated by the Japan Vam & Poval Co., Ltd. (Osaka, Japan) and Yaizu Suisankagaku Ind. (Shizuoka, Japan), respectively. HRP (190 U/mg), H_2O_2 aqueous solution (30% (w/w)), *N*-hydroxysulfosuccinimide (NHS), lactobionic acid, 3-(*p*-hydroxyphenyl) propionic acid, and tetramethylethylenediamine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Tyramine hydrochloride, 3-(4-hydroxyphenyl) propionic acid, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and the Peptide Institute (Osaka, Japan), respectively.

2.2. Synthesis of Ph-PVA and Ph-chitosan

Ph-PVA was synthesized based on a method described elsewhere (Sakai et al., 2013). Briefly, PVA-COOH and tyramine hydrochloride were each dissolved in 100 mM morpholinoethanesulfonic acid (MES) buffer (pH 6.0) at 3.1% (w/w). To this solution, NHS and EDC were sequentially added at 87 and 174 mM, respectively, and then stirred for 20 h at 40 °C. Two Ph-chitosan specimens differing in Ph content were synthesized based on a reported method (Sakai, Yamada, Zenke, & Kawakami, 2009a). Briefly, chitosan was dissolved in a 18.4 mM HCl aqueous solution at 7.6% (w/w). To this solution, tetramethylethylenediamine was added at 1.1% (w/w). After adjusting pH to 4.8, lactobionic acid and 3-(4-hydroxyphenyl) propionic acid were added at 0.04 and 1.5% (w/w), respectively. To this solution, EDC was added at 0.3 or 1.0% (w/w), and then stirred for 20 h at 40 °C. The Ph-chitosans obtained by adding 0.3 and 1.0% (w/w) EDC were denominated as PhL- and PhH-chitosan, respectively. A specimen obtained without adding 3-(4-hydroxyphenyl) propionic acid was denominated as NPh-chitosan. The resultant solution containing chitosan derivative was gradually dripped into 90% (v/v) ethanol/10% (v/v) water for precipitation of the polymer. The precipitated polymer was washed with 90% (v/v) ethanol/10% (v/v) water until an ultraviolet visible spectrum peak at 275 nm attributed to residual tyramine was undetectable in the supernatant fluid after centrifugation. The resultant polymers were then vacuum dried. The contents of Ph groups in the resultant Ph-PVA, PhL-chitosan, and PhH-chitosan measured

using ^1H NMR were 2.9×10^{-4} , 2.0×10^{-4} , and 3.9×10^{-4} mol-Ph/g, respectively.

2.3. Gelation time

Gelation time of polymer solutions obtained by dissolving Ph-chitosan and/or Ph-PVA in Krebs Ringer Hepes-buffered saline (KRH, pH 7.4) was determined based on a previously reported method (Sakai & Kawakami, 2007). A solution containing either or both of Ph-chitosan and/or Ph-PVA at 5% (w/v) total polymer content was poured into a 48-well plate at 400 μL /well. The content of each polymer is shown in Table 1. Subsequently, 100 μL of phosphate-buffered saline (pH 7.4, PBS) containing HRP and H_2O_2 was poured into each well and stirred using a magnetic stirrer bar (10-mm length $\times$ 4-mm diameter). When the magnetic stirring was hindered and the surface of the solution swelled, this was considered to signal the formation of the gel state. The final concentrations of polymer(s), HRP and H_2O_2 were fixed at 4% (w/v), 1 U/mL and 1 mM, respectively. The means and standard deviations of quadruplets were reported.

2.4. Mechanical properties

Cuboid hydrogels, (10 mm in width $\times$ 40 mm in length $\times$ 3 mm in thickness), were prepared for extension studies by pouring KRH containing Ph-chitosan and/or Ph-PVA, HRP (1 U/mL), and H_2O_2 (1 mM) at the polymer concentrations shown in Table 1 into molds. After 12 h of standing at room temperature in humidified air, the specimens taken from the molds were placed in a Table-Top Material Tester (EZ-Test; Shimadzu, Kyoto, Japan). The specimens were cut to a 20-mm length and clamped at both ends. The length between the clamps was set at 10 mm. The extension-stress profile was measured by stretching a specimen at 20 mm/min until rupture. The means and standard deviations were determined by the test using five specimens prepared separately under each condition.

2.5. Swelling study

Column-shaped hydrogels, 11 mm in diameter and 5 mm in height, were prepared by pouring the polymeric solutions shown in Table 1 at 1 U/mL HRP and 1 mM H_2O_2 into molds. After 12 h of standing at room temperature in humidified air, the hydrogels taken from the molds were soaked in 25-times larger volumes of PBS at room temperature. The changes in diameter and height of the column-shaped hydrogels were measured every day for 6 days. The swelling ratio was calculated using the following equation: swelling ratio = (volume of hydrogel after soaking in PBS)/(volume of hydrogel before soaking in PBS). Five specimens prepared separately were tested for each condition. The means and standard deviations were reported.

2.6. Fibroblastic cell behavior

Hydrogels were prepared under the conditions shown in Table 1 by pouring 0.5 mL solution to a 12-well tissue culture dish, so as to completely cover the bottom of a well before hydrogelation. After 12 h of standing at room temperature in humidified air, 6 mL of Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) with 10% (v/v) fetal bovine serum (FBS) was poured into the well and allowed to stand for 10 min. This step was repeated four times to remove remaining H_2O_2 and replace KRH with the medium. DMEM with 10% (v/v) FBS containing mouse embryo fibroblast-like cell line 10T1/2 cells (Riken Cell Bank, Ibaragi, Japan) was then poured into each well at 2×10^4 cells/well.

Table 1
Hydrogel compositions prepared through the HRP-catalyzed reaction.

	Concentration [% (w/v)]				Ph content [×10 ^{−5} mol-Ph/mL]
	Ph-PVA	Chitosan derivative			
		PhL-	PhH-	NPh-	
4Ph-PVA	4	–	–	–	1.16
1PhL-chitosan/3Ph-PVA	3	1	–	–	1.07
2PhL-chitosan/2Ph-PVA	2	2	–	–	0.98
3PhL-chitosan/1Ph-PVA	1	3	–	–	0.89
4PhL-chitosan	–	4	–	–	0.80
4PhH-chitosan	–	–	4	–	1.56
2PhH-chitosan/2Ph-PVA	2	–	2	–	1.36
2NPh-chitosan/2Ph-PVA	2	–	–	2	0.58
2Ph-PVA	2	–	–	–	0.58

At 14 h after cell seeding, adhesion of the cells on hydrogels was observed using an optical microscope.

2.7. *E. coli* behavior

Hydrogels were prepared under the conditions shown in Table 1 in a plastic petri dish (35 mm in diameter) by pouring 1.5 mL of mixture solution before hydrogelation. After 12 h of standing at room temperature in humidified air, 10 mL of Lysogeny Broth (LB) medium was poured into the dish and allowed to stand for 30 min. After repeating the step four times to replace the solution in hydrogel to LB medium, the dish was turned out onto a sterilized paper to remove the remaining medium from the hydrogel. Fifty microliters LB medium containing *E. coli* MM294 cells pre-cultured for 1 day in an Erlenmeyer flask (1 L) containing 200 mL medium at 37 °C using a reciprocal shaker (100 rpm, 4 cm amplitude) was then spread on each hydrogel using a spreader. Colony development was evaluated after 48 h of incubation at 37 °C.

3. Results and discussion

3.1. Hydrogelation

An attractive feature of HRP-catalyzed hydrogelation is the mild reaction conditions that are suitable for mammalian cells. A potential application of Ph-chitosan/Ph-PVA hydrogels obtained through a cell-friendly hydrogelation system is injectable scaffolds for tissue engineering, tissue-reconstructions, including wound healing, and drug delivery (Teixeira et al., 2012). For these applications, knowledge of the effect of the contents of Ph-chitosan and Ph-PVA on the time taken for hydrogelation (gelation time) is important. As shown in Fig. 2, the content of each polymer influenced the gelation time. At 4.0% (w/v) total polymer concentration of either or both of Ph-PVA and/or PhL-chitosan, gelation time decreased with increasing PhL-chitosan content. The longest and shortest gelation times were detected at 4% (w/v) Ph-PVA and 4% (w/v) PhL-chitosan, respectively. Focusing on the Ph content, the trend obtained for the Ph-PVA and PhL-chitosan system was that the gelation time decreased with decreasing Ph group content (Fig. 2, Table 1). However, the opposite trend was observed for single-component solutions of 4Ph-PVA and 2Ph-PVA in this study and our previous results, including Ph-chitosan (Sakai, Yamada, et al., 2009; Sakai, Hirose, Taguchi, Ogushi, & Kawakami, 2009b). The independence of the content of Ph groups was also shown by shorter gelation times of 2PhH-chitosan/2Ph-PVA solution with the higher content of Ph groups compared with that of 2PhL-chitosan/2Ph-PVA. These findings indicate that the gelation time of a mixed system can be estimated from the times taken for gelation of solutions containing a single component, but not from the content of Ph groups in the solution.

3.2. Mechanical properties and swelling in PBS

Blending of PVA with chitosan has been used to improve the mechanical properties of matrices such as films and hydrogels containing chitosan (Chen, Liu, Huang, Wang, & Fang, 2013; Liang et al., 2009; Takei et al., 2013). We investigated the effect of conjugation by the HRP-catalyzed reaction on the mechanical properties of the resultant hydrogels by measuring the stresses and breaking points under tensile strain (stretching). As shown in Fig. 3, the 4PhL-chitosan hydrogel broke at ca. 50% strain, while the breaking point for 4Ph-PVA hydrogel occurred at ca. 150% strain. The strain at rupture for PhL-chitosan/Ph-PVA conjugate hydrogels varied between the values obtained for 4PhL-chitosan and 4Ph-PVA and the value increased with increasing Ph-PVA content. These results indicate that conjugation through the HRP-catalyzed reaction was effective for improving the brittleness of Ph-chitosan hydrogels, likewise in the case of hydrogels prepared by non-enzymatic cross-linking methods (Chen et al., 2013; Liang et al., 2009; Takei et al., 2013). Regarding the stress at rupture, the value for 1PhL-chitosan/3Ph-PVA was larger than those for 4PhL-chitosan and 4Ph-PVA. This result indicates that conjugation is effective for obtaining hydrogels with superior durability toward deformation, which cannot be predicted from the results for the hydrogels composed of Ph-PVA or Ph-chitosan alone. Regarding the effect of the Ph content in Ph-chitosan, a more brittle hydrogel was obtained from Ph-chitosan with a higher content of Ph groups. This result may be explained by the hindrance of deformation of the highly

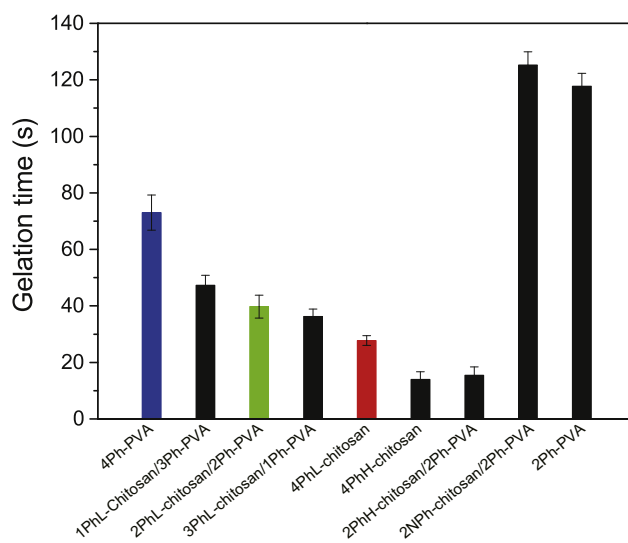


Fig. 2. Gelation times for polymeric solutions of differing composition. Values represent the mean ($n = 4$) with standard deviation.

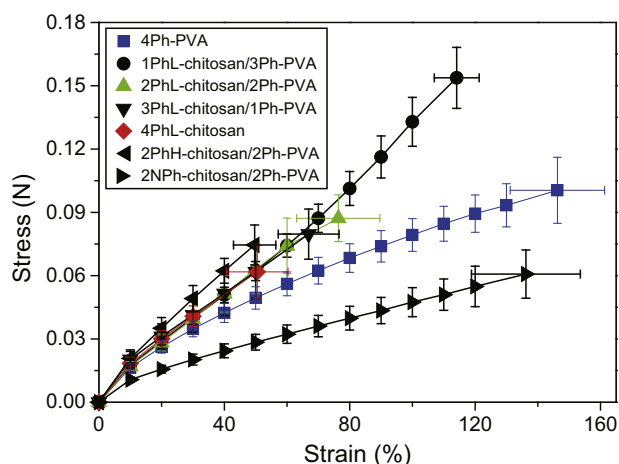


Fig. 3. Strain–stress profiles of hydrogels obtained from polymeric solutions of differing composition. Values represent the mean ($n=5$) with standard deviation.

cross-linked network under mechanical stress, and the faster gelation rate of 2PhH-chitosan/2Ph-PVA than those for 2PhL-chitosan/2Ph-PVA and 2NPh-chitosan/2Ph-PVA (Fig. 2) inducing a less uniform microscopic structure. The formation of hydrogels showing lower resistance to deformation under conditions giving faster hydrogelation was reported for a variety of hydrogels obtained through non-enzymatic cross-linking methods, such as alginate (Kuo & Ma, 2001) and collagen hydrogels (Achilli & Mantovani, 2010).

For potential applications of Ph-chitosan/Ph-PVA hydrogels to the biomedical field, knowledge of their swelling properties in saline is important. We investigated this property by measuring the volume change of hydrogels. 4Ph-PVA hydrogel did not swell markedly during 6 days of soaking in PBS (Fig. 4). In contrast, the degree of swelling of 4PhL-chitosan hydrogel was the largest amongst the hydrogels examined in this study. The degree of swelling decreased with increasing Ph-PVA content in 4% total-polymer hydrogels. This finding may be attributed to the formation

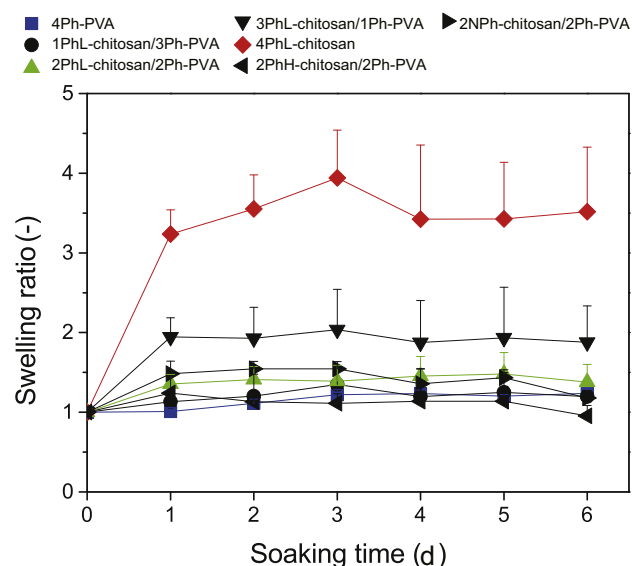


Fig. 4. Swelling profiles of hydrogels obtained from polymeric solutions of differing composition. Values represent the mean ($n=5$) with standard deviation.

of H-bonds between the amino groups in Ph-chitosan and the OH-groups in Ph-PVA (Abdel-Mohsen, Aly, Hrdina, Montaser, & Hebeish, 2011). A less homogeneous microscopic structure of the hydrogel with the higher content of PhL-chitosan induced by faster hydrogelation may also be a reason for this behavior (Fig. 2). The same tendency was reported for chitosan/PVA hydrogels obtained through non-enzymatic cross-linking methods such as repeated freezing–thawing (Abdel-Mohsen et al., 2011), ultraviolet irradiation (Kim, Park, & Kim, 2003), and gamma ray irradiation (Tahtat et al., 2011).

3.3. Adherent mammalian cells and *E. coli* cell behaviors

Chitosan (Chandy & Sharma, 1990; Jarmila & Vavrikova, 2011) and PVA (Suci et al., 2004) have been investigated as promising

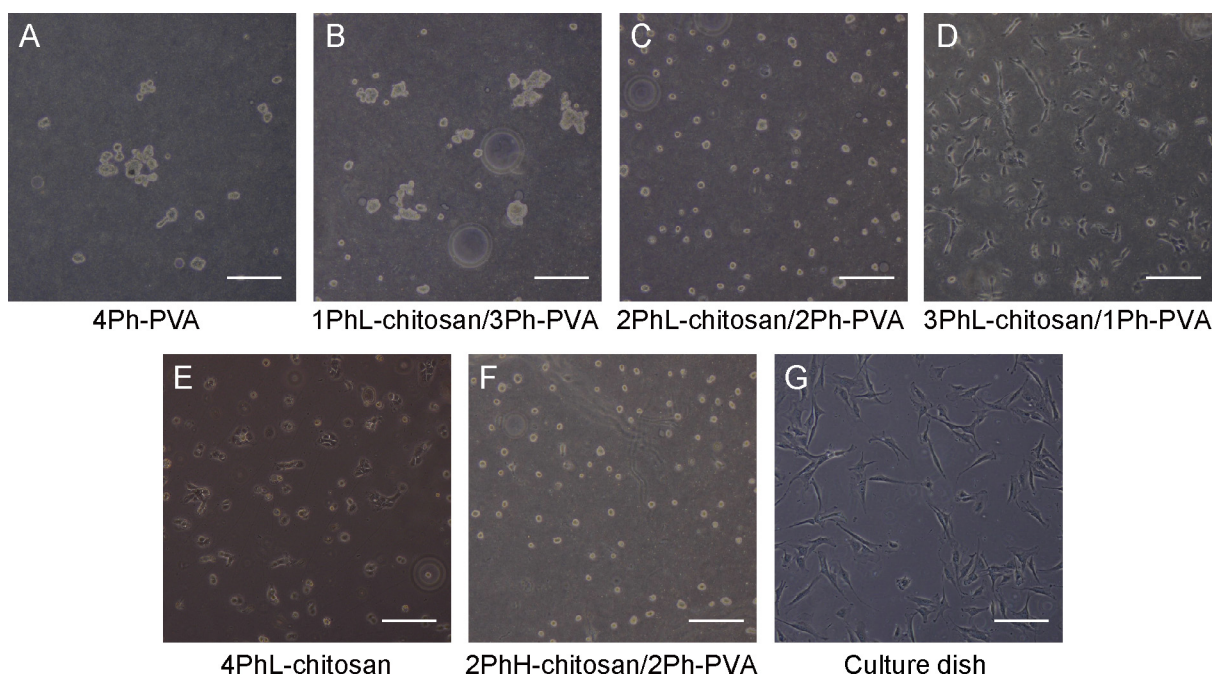


Fig. 5. Microphotographs of 10T1/2 cells 14 h after seeding on hydrogels obtained from polymeric solutions of differing composition. Bars: 100 μm .

materials for biomedical applications. To evaluate the usefulness of bioconjugated Ph-chitosan/Ph-PVA hydrogels in the biomedical field, we examined fibroblastic cell adhesion and antibacterial properties of Ph-chitosan/Ph-PVA hydrogels prepared under different conditions. Fig. 5 shows 10T1/2 cells 14 h after seeding onto hydrogels composed of either or both of Ph-chitosan and/or Ph-PVA. The cells did not adhere onto 4Ph-PVA (Fig. 5A) or 1PhL-chitosan/3Ph-PVA (Fig. 5B) hydrogels and formed clusters. The cells on 2PhL-chitosan/2Ph-PVA adhered but did not spread (Fig. 5C). Spreading of the cells was induced by increasing the content of PhL-chitosan in 3PhL-chitosan/1Ph-PVA (Fig. 5D), but the degree of spreading was lower than those on a cell culture dish (Fig. 5G). Further increase in the content of PhL-chitosan, i.e., 4PhL-chitosan hydrogel, decreased the degree of spreading (Fig. 5E). Difficulty in adhesion and spreading of adherent cells has been reported for chitosan and chitosan derivative hydrogels obtained through non-enzymatic cross-linking methods (Ono et al., 2000; Takei, Nakahara, Ijima, & Kawakami, 2012). A greater spreading of 10T1/2 cells on 2PhL-chitosan/2Ph-PVA hydrogel than those on 4PhL-chitosan hydrogel may be induced by the moderate cationic property resulting from the conjugation with an appropriate amount of Ph-PVA. These results suggest the possibility of controlling cell adhesion on the hydrogels

by altering the content of PhL-chitosan and Ph-PVA for an intended application.

Antibacterial properties are one of the attractive features of chitosan for applications in the biomedical field (Jarmila & Vavrikova, 2011; Jarry et al., 2001; Tahtat et al., 2011). We evaluated the biocidal impact of Ph-chitosan/Ph-PVA hydrogels on *E. coli* cells. Fig. 6 shows the hydrogels 48 h after seeding *E. coli* cells. Interestingly, the hydrogel containing PhL-chitosan at 2% (w/v) or more suppressed the growth of *E. coli* (Fig. 6C–E). This result can be explained by the reported mechanism of antibacterial action by chitosan caused by the formation of electrostatic bonds between chitosan and anionic groups on the bacterial surface membrane, which would result in altered membrane permeability (Jarmila & Vavrikova, 2011; Tsai & Su, 1999; Yang, Liu, Chen, Yu, & Zhu, 2008). In our specimens, it is intuitive that hydrogels with an abundance of Ph-chitosan show greater cationic properties. The growth of *E. coli* on 2PhH-chitosan/2Ph-PVA (Fig. 6F) but not on 2PhL-chitosan/2Ph-PVA (Fig. 6C) can also be explained by the difference in the degree of cationic strength. The degree of substitution of amino groups in PhL-chitosan was less than that in PhH-chitosan. These results indicate that the content and degrees of modification of Ph-chitosan in Ph-chitosan/Ph-PVA hydrogels should be carefully chosen for applications in which antibacterial properties are desired.

4. Conclusions

Ph-chitosan/Ph-PVA hydrogels were prepared through the HRP-catalyzed cross-linking between Ph groups at different Ph-chitosan and Ph-PVA contents. Gelation time was dependent on the content of each polymer, but independent of the content of Ph groups in the solution. At 4.0% (w/v) total polymer concentration of either or both of Ph-PVA and/or PhL-chitosan, gelation time decreased with increasing Ch-PhL content. The longest and shortest gelation times detected for 4% (w/v) Ph-PVA and 4% (w/v) PhL-chitosan were 73 s and 28 s, respectively. Swelling of the hydrogels can be suppressed by increasing the Ph-PVA content. The swelling ratio of 3PhL-chitosan/1Ph-PVA hydrogel was 1.9 at 6 days of soaking in PBS. The value decreased to 1.2 at 1PhL-chitosan/3Ph-PVA hydrogel. Fibroblastic cell adhesion and antibacterial activity studies demonstrated that these bioactivities can be controlled by altering the Ph-chitosan and Ph-PVA content in the hydrogels. To obtain hydrogels with greater cell adhesiveness, it was shown that an appropriate Ph-chitosan and Ph-PVA content is necessary. The cells on 3PhL-chitosan/1Ph-PVA hydrogel were the most spreading compared to those on 2PhL-chitosan/2Ph-PVA and 4PhL-chitosan hydrogels. To obtain the hydrogels with greater antibacterial activity, it was shown that a higher Ph-chitosan content is necessary. *E. coli* cells grew on 1PhL-chitosan/3Ph-PVA hydrogel but did not grow on 2PhL-chitosan/2Ph-PVA hydrogel during 48 h cultivation. These results demonstrate the feasibility of HRP-catalyzed hydrogelation for obtaining the hydrogels having the unique properties attributed to the conjugation of Ch-chitosan and Ch-PVA which cannot be obtained using either of the components alone.

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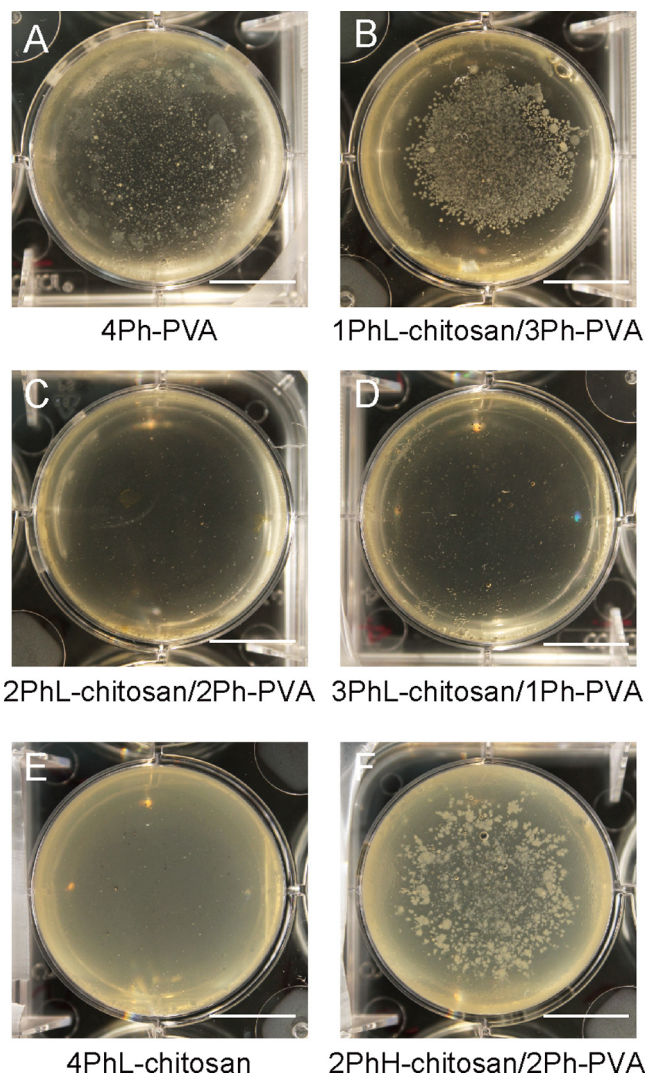


Fig. 6. Photographs of hydrogels obtained from polymeric solutions of differing composition 48 h after seeding *E. coli* cells. Bars: 1 cm.

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