Tissue Adhesive Properties of Functionalized Chitosan: A Comparative Study of Phenol, Catechol and Gallol

Yasushi Hino and Hirotaka Ejima*

Department of Materials Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan
*ejima@material.t.u-tokyo.ac.jp

Tissue adhesives are greatly employed as surgical sealants for wound-healing patches and hemostatic dressing. However, achieving strong adhesion of wet tissues is still challenging because the bonding strength of biomedical adhesives is generally weakened by physiological fluids due to their high dielectric and ionic strengths. To overcome this challenge, bioinspired approaches have been adopted to enhance the wet adhesion of biocompatible polymers. Here, we synthesized a series of chitosan functionalized with bioinspired phenolic groups, i.e., phenol, catechol, and gallol, with similar modification rates. The bonding and sealing strengths of wet tissues by this functionalized chitosan were compared under similar conditions. We observed that the gallol group was the most effective in enhancing the tissue adhesion property of chitosan.

Keywords: Surgical glue, Biomimetics, Mussel, Tunicate, Marine organisms

1. Introduction

It is estimated that over 300 million surgeries were performed globally in 2012 [1]. During surgeries, sutures, staples, and clips are commonly used for wound closure [2]. These traditional devices are mechanically reliable, although their inability to prevent air and fluid leakages often results in secondary tissue damages and microbial infections [3]. To overcome these problems, tissue adhesives based on fibrins, polyethylene glycols, cyanoacrylate, polypeptides, and polysaccharides are employed as adjuncts with sutures to seal and repair wounds [2].

Chitosan is a naturally occurring polysaccharide that possesses wound-healing, biodegradable, biocompatible, antibacterial, and hemostatic properties [4]. Chitosan is obtained by the partial deacetylation of chitin, which is a naturally abundant component of the exoskeletons of crustaceans [5]. The primary amine groups of chitosan enable its facile functionalization by carboxy-bearing compounds via condensation reactions. Ono et al. [6] introduced azide and lactose moieties via a two-step condensation reaction to yield photocrosslinkable chitosan. The material glued two slices of ham with a bonding strength of 4.2 kPa, which was slightly larger than that of commercial fibrin glue (4.0 kPa). Despite these advances, the bursting pressure was still lower than the systolic blood pressure (ca. 16 kPa) [7]. The sealing of wet tissues is still challenging because adhesion is generally weakened by physiological fluids due to their high dielectric and ionic strengths [8]. To enhance the bonding strength of chitosan-based adhesives in wet tissues, the biomimetic approach is effective because some marine organisms can stick even in seawater [9].

Marine mussels can firmly attach themselves to various substrates under wet conditions, while most synthetic glues cannot [10]. The key to their underwater adhesion is the utilization of unique catechol-functionalized proteins in their byssal threads [11]. Inspired by mussels’ adhesion strategy, Ryu et al. [12] developed catechol-functionalized chitosan. When complexed with thiolated pluronic F-127 for crosslinking, it exhibited a bonding strength of 15.0 ± 3.5 kPa, whereas the negative control experiment without catechol exhibited a significantly lower bonding strength (5.3 ± 2.6 kPa). Sanandiya et al. [13] prepared gallol-functionalized chitosan possessing...
one more hydroxyl group than the catechol-functionalized chitosan. The bonding strength in porcine skin approached 47 kPa according to the results of lap shear tests. These results clearly demonstrated that the introduction of the phenolic moieties, i.e., catechol or gallol, significantly enhanced the wet adhesion of chitosan. However, it is still unclear how the number of phenolic hydroxyl groups influences the adhesion strength under the same experimental condition.

Here, we synthesized a series of chitosan compounds, functionalized with bioinspired phenolic groups (phenol, catechol, and gallol) with similar modification rates (ca. 15%). Using these model polymers, the bonding and sealing strengths of wet tissues were systematically investigated under identical conditions. We discovered that the gallol group was the most effective of all in enhancing the tissue adhesion of chitosan.

![Fig. 1. Synthesis scheme of phenol-functionalized chitosan (CS-Phe), catechol-functionalized chitosan (CS-Cat), and gallol-functionalized chitosan (CS-Gal).](image)

2. Experimental
2.1. Materials
Chitosan 10 (viscosity-average molecular weight, \( M_V = 2.1 \times 10^5 \)) [14], 1-hydroxybenzotriazole (HOBt), 3,4,5-trihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl), 2-(N-morpholino) ethanesulfonic acid (MES), and sodium nitrite were purchased from Wako Pure Chemical Industries, Ltd. Sodium molybdate was obtained from Sigma-Aldrich.

2.2. Synthesis of functionalized chitosan
Chitosan was functionalized using phenolic groups according to a previously reported procedure [15] with minor modifications. Chitosan (2.00 g) and HOBt (1.60 g) were added to distilled water and stirred until the solution became clear. Thereafter, 3,4,5-trihydroxybenzoic acid (2.01 g), 3,4-dihydroxybenzoic acid (1.82 g), or 4-hydroxybenzoic acid (1.63 g) was added to the solution. Next, EDC-HCl (2.26 g) in ethanol (3 mL) was added dropwise to the solution. The solution was stirred overnight at a room temperature. The resultant solution was dialyzed in an MES buffer (17 mM, pH 4.5–5.0) containing 150 mM NaCl for 3 days, and afterward, in distilled water for 8 h using a dialysis membrane (Spectra/Por 7, MWCO 50 kDa, Repligen). Thereafter, the solution was lyophilized.

To quantitate the degree of functionalization, a modified Arnow’s assay [16,17] was performed. The functionalized chitosan (10 mg) was dissolved in 5 mL of distilled water. Afterward, 1 mL of 0.1 M HCl, 1 mL of Arnow’s reagent (10% w/v sodium molybdate and 10% w/v sodium nitrite), and 1 mL of 1 M NaOH were added. The absorbance at 520 nm was recorded three times for each sample. Linear standard curves were constructed separately for the phenolic compounds, namely 3,4,5-trihydroxybenzoic acid, 3,4-dihydroxybenzoic acid, or 4-hydroxybenzoic acid. The phenolic content of the functionalized chitosan was estimated using the standard curves.

2.3. Measurement of the binding strength
The shear strength was measured by lap shear tests based on the American Society for Testing and Materials (ASTM) F2255-05 with minor modifications. Two pieces of pork skin (20 × 40 mm and 4 ± 1 mm thick) were overlapped. An aqueous solution (60 mg) of an adhesive polymer (14 mg, ca. 23 wt%) was applied to the overlapping areas (4 cm²) to glue the pieces. After a certain curing period, the samples were pulled apart at a rate of 10 mm min⁻¹. The separation force was recorded by a tensile testing machine (MCT-2150, A&D). The binding strength was calculated by dividing the bond failure force by the overlapped area.

The adhesion strength was measured by subjecting the glued pieces to a wound closure test based on ASTM F2458-05 with minor modifications. A piece of pork skin (20 × 60 mm and 4 ± 1 mm thick) was cut in the middle using a pair of scissors, to simulate wounding. The two pieces of pork skin (20 × 30 mm each) were placed side-by-side in such a way that the 20 mm sides were in contact. An aqueous solution (60 mg) of the adhesive polymers (14 mg, ca. 23 wt%) was
applied to the boundary between the two pieces. After a certain curing period, the samples were pulled apart at a rate of 10 mm min\(^{-1}\) using a tensile testing machine (MCT-2150, A&D). The separation force was recorded. The binding strength was calculated by dividing the bond failure force by the contact area.

2.4. Measurement of the sealing strength

The sealing strength was measured by subjecting the glued sample to a burst pressure test based on ASTM F2392-04 with minor modifications. Further, a pinhole (3 mm in diameter) was punched in the middle of the bovine aorta (40 × 40 mm and 4 ± 1 mm thick). An aqueous solution (60 mg) of the adhesive polymer (23 wt%) was dropped into the pinhole. The sealed sample was afterward, placed in a custom-made burst pressure apparatus. Phosphate-buffered saline (PBS) was pumped and the pressure required to burst the seal of the pinhole was measured using a pressure gauge (KDM30-500 kPa, Krone).

3. Results and discussion

3.1. Synthesis and characterization of phenolic-functionalized chitosan

The phenolic molecules, namely 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, and 3,4,5-trihydroxybenzoic acid, were conjugated to the amino groups of chitosan to produce CS-Phe, CS-Cat, and CS-Gal, respectively (Fig. 1). The unreacted phenolic molecules were thoroughly removed by dialysis. In the \(^1\)H NMR spectra (Fig. 2), the characteristic peaks at 7–8 ppm, which can be attributed to the phenolic protons of the functionalizing groups appeared.

To determine the most effective phenolic group in enhancing the adhesion properties of chitosan, the modification rate was set at a similar range. The degree of deacetylation of the chitosan employed in this study was 82% as obtained from \(^1\)H NMR spectroscopy. The degrees of phenolic-functionalization \((n)\) in Fig. 1) estimated by integrating the \(^1\)H NMR peaks were 0.16, 0.14, and 0.13 for CS-Phe, CS-Cat, and CS-Gal, respectively. These values were verified by Arnow’s assay [16,17], whose degrees of 0.17, 0.13, and 0.15 for CS-Phe, CS-Cat, and CS-Gal, respectively, correlated. Thus, the modification rates for all the phenolic compounds were in the range of \(n = 15 \pm 2\%\), allowing us to compare the effects of the bioinspired phenolic groups under almost the same conditions.

Fig. 2. The \(^1\)H NMR spectra of chitosan in DCI/D\(_2\)O (5%, v/v) and those of the functionalized chitosan (CS-Phe, CS-Cat, and CS-Gal) in D\(_2\)O.

3.2. Adhesion strength of the functionalized chitosan

We examined three critical strengths of tissue adhesion (shear strength, adhesive strength, and burst pressure) according to ASTM standards for biological adhesives. The lap shear strengths at various curing periods are plotted in Fig. 3. In this test, two pieces of pork skin were bonded at a 4 cm\(^2\) overlapping area. The wound closure test was simulated on a scission wound (Fig. 4). In the test, the pork skin was cut into two and thereafter, glued. In these measurements, both the shear and adhesive strengths were observed to have increased with increasing curing time. The lap shear strength peaked at ~18 h, while the adhesive strength peaked at ~6 h. This is probably due to the difference in the exposed areas between the geometries of the two pieces. In the lap shear test, most of the adhesive was concealed by the pork skin, hence, the slow evaporation of the solvent. Additionally, the phenolic groups, including catechol and gallol, were gradually oxidized and covalently cross-linked on contact with oxygen [18]. It was observed that the curing time may be further reduced by increasing temperature or by irradiation. However, that is not the scope of this paper.
The shear and adhesive strengths of the phenolic polymers after 24 h were in the order: CS-Gal > CS-Cat > CS-Phe ≈ Chitosan, suggesting that the functional moiety bearing more phenolic hydroxyl groups is more effective in enhancing the adhesion than those bearing less. Moreover, a minimum of two hydroxyl groups at adjacent positions are necessary to enhance the adhesion strength because CS-Phe exhibited the same adhesion strength as chitosan. This finding correlates with our previous results from a study on poly (butyl acrylate)-based underwater adhesives [19]. The shear and adhesive strengths of CS-Gal approached 154 ± 11.6 and 353 ± 13.2 kPa, respectively, which are larger than those of commercial surgical glues, such as Evicel and CoSeal [20].

Next, the burst pressure was measured to evaluate their suitability as surgical sealants. A solution of the adhesive polymer was applied to seal a pinhole (3 mm in diameter) in the bovine aorta (as a biological substrate). The sealed tissue was afterward, placed in a custom-made burst pressure apparatus (Fig. 5). PBS was continuously pumped into the apparatus and the pressure required to burst the adhesive seal of the pinhole was recorded. Our results establish that the burst pressure increased with increasing curing time and peaked at ~6 h, similar to the observation from the wound closure test (Fig. 4). Like the shear and adhesive strengths, burst pressures are also in the order: CS-Gal > CS-Cat > CS-Phe ≈ Chitosan. The burst pressure of CS-Gal after 24 h was 207.6 ± 43.2 kPa, which was substantially higher than that of systolic blood pressure (ca. 16 kPa) [7].

4. Conclusion

In this study, series of chitosan functionalized with phenolic molecules (phenol, catechol, and gallol) were synthesized. The characterizations by 1H NMR and Arnow’s assay demonstrated that the degree of functionalization was 15 ± 2%. The tissue bonding strengths of the synthesized polysaccharides were verified by three distinct tests, namely lap shear, wound closure and burst pressure tests. In all the tests, CS-Gal demonstrated the strongest adhesion strength among the tested. The present study may promote the functionalization of polymers with gallol groups to develop strong biomedical adhesives.
Acknowledgments

This research was partially supported by the Japan Society for the Promotion of Science (JSPS) through KAKENHI; Grant Numbers 18K14000 and 18K18802, and by the Japan Agency for Medical Research and Development (AMED) through the Translational Research program; Strategic PRomotion for practical application of INnovative medical Technology (TR-SPRINT). Mizuho Foundation for the Promotion of Sciences and the Ogasawara Foundation for the Promotion of Science & Engineering are acknowledged for their financial supports. H. E. thanks JSPS for its Leading Initiative for Excellent Young Researchers (LEADER).

References

1. T. G. Weiser, A. B. Haynes, G. Molina, S. R. Lipsitz, M. M. Esquivel, T. Uribe-Leitz, R. Fu, T. Azad, T. E. Chao, and W. R. Berry, Lancet, 385 (2015) S11.
2. L. Al-Mubarak and M. Al-Haddab, J. Cutan. Aesthet. Surg., 6 (2013) 178.
3. V. Bhagat and M. L. Becker, Biomacromolecules, 18 (2017) 3009.
4. J. F. Prudden, P. Migel, P. Hanson, L. Friedrich, and L. Balassa, Am. J. Surg., 119 (1970) 560.
5. K. Kurita, Mar. Biotechnol., 8 (2006) 203.
6. K. Ono, Y. Saito, H. Yura, K. Ishikawa, A. Kurita, T. Akaike, and M. Ishihara, J. Biomed. Mater. Res., 49 (2000) 289.
7. Y. Hong, F. Zhou, Y. Hua, X. Zhang, C. Ni, D. Pan, Y. Zhang, D. Jiang, L. Yang, and Q. Lin, Nat. Commun., 10 (2019) 2060.
8. B. P. Lee, P. B. Messersmith, J. N. Israelachvili, and J. H. Waite, Annu. Rev. Mater. Res., 41 (2011) 99.
9. D. W. R. Balkenende, S. M. Winkler, and P. B. Messersmith, Eur. Polym. J., 116 (2019) 134.
10. J. H. Waite, Int. J. Adhes. Adhes., 7 (1987) 9.
11. J. H. Waite and M. L. Tanzer, Science, 212 (1981) 1038.
12. J. H. Ryu, Y. Lee, W. H. Kong, T. G. Kim, T. G. Park, and H. Lee, Biomacromolecules, 12 (2011) 2653.
13. N. D. Sanandiya, S. Lee, S. Rho, H. Lee, I. S. Kim, and D. S. Hwang, Carbohydr. Polym., 208 (2019) 77.
14. K. Ohkawa, Y. Takahashi, M. Yamada, and H. Yamamoto, Macromol. Mater. Eng., 286 (2001) 168.
15. M. Xie, B. Hu, Y. Wang, and X. Zeng, J. Agric. Food Chem., 62 (2014) 9128.
16. L. E. Arnow, J. Biol. Chem., 118 (1937) 531.
17. A. Matkowski, S. Zielińska, J. Oszmiański, and E. Lamer-Zarawska, Bioresour. Technol., 99 (2008) 7892.
18. M. Rahimnejad and W. Zhong, RSC Adv., 7 (2017) 47380.
19. K. Zhan, C. Kim, K. Sung, H. Ejima, and N. Yoshie, Biomacromolecules, 18 (2017) 2959.
20. E. S. Sani, A. Kheirkhah, D. Rana, Z. Sun, W. Foulsham, A. Sheikhi, A. Khademhosseini, R. Dana, and N. Annabi, Sci. Adv., 5 (2019) eaav1281.