Effects of crosslinking methods on network structure and enzymatic degradation of methacrylate-functionalized chitosan hydrogel

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Abstract

Polysaccharides, such as hyaluronic acid, alginate, or chitosan, can be modified by addition of reactive functional groups to enable chemical crosslinking. Here, we studied how different methods of crosslinking methacrylate-functionalized chitosan affected the network structures of the resulting hydrogels. We then investigated how the porous network structures in turn influenced stiffness, macromolecular diffusion through the pores, and enzymatic degradation. All these properties are relevant for utilization of the chemically crosslinked hydrogels in biomedical applications, including tissue engineering and delivery of therapeutic agents. We made chitosan hydrogels using four crosslinking methods, which differ by type and by reaction kinetics. We found that four chitosan hydrogels having identical polymer fractions at an equilibrium swelling exhibited marked differences in their shear moduli, rate of dextran diffusion, and especially their enzymatic degradation behaviors. We inferred that these differences originated in variations among network structures, which were characterized by the formation of chain bundles and associated network heterogeneity as determined by small-angle X-ray scattering analysis.

Hydrogels composed of polysaccharides, such as hyaluronic acid, alginate, or chitosan, exhibit enzymatic degradability and low cytotoxicity. As a consequence of such properties, these hydrogels are used extensively as primary materials for various biomedical applications, such as therapeutic agent delivery, tissue engineering, medical implants, and in vitro cell culture1-5. Because materials used in these fields frequently require enhanced mechanical properties and/or spatiotemporal control over their formation, a number of synthetic strategies have been introduced6-15 to enable chemical crosslinking of the polysaccharides, which can potentially enhance their usefulness as engineered biomaterials. Chemically crosslinked polysaccharide hydrogels are often generated through polymerization of macromers, which are pre-polymerized chains tethered by one or more polymerizable groups, such as (meth)acrylate6, 11, 14, 16, (meth)acrylamide6, 15, maleimide9, 12, or norbornene13. To be used as engineered biomaterials, these hydrogels should possess desired properties at requisite levels, such as enzymatic degradability, transport of macromolecules through the pores, mechanical properties, and equilibrium water content.

These properties are determined primarily by the structural characteristics of hydrogel networks, which include crosslinking density, average pore size, pore size distribution, and heterogeneity of network density at varying lengths7, 10, 16-19(11). For a macromer with a fixed chemistry, these structural properties are determined solely by the type and reaction kinetics of the crosslinking method17-18. Despite the critical role of the crosslinking method, a limited number of studies have focused on the effects of polysaccharide-based macromer crosslinking methods on properties that are relevant for employing chemically crosslinked hydrogels in biomedical applications.

Chitosan is degraded in vivo by a lysozyme present in human tears, saliva, and mucus. It has been used as an engineered biomaterial for ocular drug delivery and bone tissue engineering11, 20-23. In the present study, we used chitosan as a model polysaccharide. We synthesized chitosan hydrogels from methacrylate-functionalized
chitosan macromers using various crosslinking methods that differed by type and crosslinking reaction kinetics. We examined how the different crosslinking methods affected properties of the network structures and other relevant features, such as stiffness, macromolecular transport, and enzymatic degradation. Polymer fractions of all investigated hydrogels were identical in both pre-gel solutions and the resulting hydrogels at swelling equilibrium.

Figure 1 illustrates the various methods employed to generate chitosan hydrogels from chitosan macromer modified with methacrylate groups (CS-GMA). Chitosan hydrogels formed by photo-initiated free radical polymerization (FRP) were given the designation RP, and the subscripts L and H were used to indicate whether low or high UV intensity was used to initiate FRP. The subscript CT indicates use of the chain-transfer agent (CTA) dithiothreitol (DTT) during FRP. Chitosan hydrogels formed by step polymerization via a base-catalyzed thiol-ene reaction between methacrylate groups and DTT molecules were termed SP.

![Figure 1](image1.png)

**Figure 1.** Synthetic routes for chitosan hydrogel generation and schematic illustrations of the expected network structures of RP_L and RP_H (left), or RP_CT and SP (right). Open circles represent the point at which the chain moves out of the plane.

Figure 1 also shows the predicted network structures of the chitosan hydrogels. In RP_L and RP_H, chain bundles were expected to form by sequential linkage of methacrylate groups in different macromers, which was indicated as each crosslink having a high degree of polymerization (DP). The average distance, \( d_{ma} \), between methacrylate groups in a semi-dilute solution of CS-GMA was roughly estimated to be \( \sim 1.7 \) nm by \((V/n)^{\frac{1}{3}}\), where \( V \) and \( n \) were the volume of the solution and the total number of methacrylate groups on CS-GMA, respectively. Because \( d_{ma} \) is an order of magnitude longer than a single C-C bond (0.15 nm), we expected that formation of chain bundles would be accompanied by local segregation of chitosan chains. Therefore, we predicted the presence of low-density regions between chain bundles characterized by weakly bound chitosan chains\(^{24-26}\).

In contrast, we predicted the average DP at crosslinks in RP_CT and SP would be much lower than in RP_L and RP_H, as equimolar amounts of thiol and methacrylate groups were present. This would indicate much less segregation among chitosan chains in RP_CT and SP. Because we used almost equimolar amounts of DTT (\( pK_a = 9.6 \)) and the base catalyst (\( pK_a = 13.5 \)) for SP synthesis, we expected each deprotonated thiolate anion to react with a single methacrylate group on average, which would yield an average DP of one. On the other hands, the molar ratio of the photo-initiator to methacrylate groups for synthesis of RP_CT was only 0.02. Moreover, the thiol chain-transfer constant, defined as the ratio of the rate constant of monomer propagation to that of the chain-transfer to thiol during the FRP of methacrylate, is typically \(<1 \)\(^{27}\). Hence, the average DP of RP_CT was expected to be greater than that of SP, although it would be much smaller than DP of RP_L and RP_H.

Equal amounts of the photo-initiator as well as identical UV intensity and irradiation times were used for the FRP of RP_H and RP_CT. However, based on the Raman scattering intensity at 1635 cm\(^{-1}\) corresponding to unreacted methacrylate groups in each hydrogel\(^{28}\) (Figure 2A), we found that a significant portion of the
methacrylate groups in RP_H had not reacted, whereas the proportion of unreacted methacrylate groups in RP_CT was negligible. We inferred that such low methacrylate conversion in RP_H was mainly due to the limited and slower motion of chitosan chains located in more highly entangled regions, i.e. chain bundles, which reduced the number of methacrylate groups spatiotemporally accessible to the growing chain ends. Additional UV exposure for 60 seconds resulted in almost complete conversion of methacrylate groups in RP_H.

To more quantitatively examine network structures of chitosan hydrogels, we used the small-angle X-ray scattering (SAXS) analysis. In Figure 2B, we found that the scattering intensity for all four hydrogels was accompanied by an upturn at a low q region. Such feature indicates that the network structures can be described by a combination of polymer concentration fluctuations at the high q region and density differences of static inhomogeneities at the low q region, which are represented by the Lorentzin-type function and the Debye-Bueche function, respectively. However, we found that a large uncertainty was associated with estimating the characteristic length of inhomogeneities because we only observed a constant slope of -4 at a low q region, indicating that the characteristic length of inhomogeneities was too large to be probed by the SAXS analysis. Consequently, we employed a combined equation of the approximated Debye-Bueche function and the Lorentzian type function to predict the scattering intensity I(q)

\[ I(q) = \frac{A}{q^4} + \frac{B}{(1+(q\xi)^m)} \]  

where A, B, m and \( \xi \) are adjustable parameters and represent scaling factors of each function, the exponent, and the correlation length, respectively. When m=2, the second term represents the Ornstein-Zernike (OZ) function, which have described semi-dilute solutions of ideal chains with \( \xi \) describing a characteristic length for the interaction with neighboring chains. RP_CT and SP were well fitted to the equation (1) with m=2, which indicated that these hydrogels have a typical network structure consisting of Gaussian chains. The correlation lengths of RP_CT and SP were 4.5 nm and 5.5 nm, respectively, which can be considered as an average mesh size of the networks. However, the best-fit values of m for RP_L and RP_H were 2.5 (Table 1). The exponent m in the range of 2 to 3 indicates a mass fractal of branched and globular structures. The results suggest that there are regions characterized by branched and globular structures in RP_L and RP_H, which we infer to be chain bundles.

| Table 1. SAXS fit parameters of four chitosan hydrogels |
|-------------|--------|--------|--------|--------|
| Fit parameter | RP_L  | RP_H  | RP_CT | SP     |
| A (x 10^-7)    | 5.25±0.01 | 6.49±0.01 | 8.09±0.01 | 5.25±0.01 |
| B               | 548±1    | 471±1   | 255±1  | 75±1   |
| \( \xi \) (Å)  | 67.1±0.1 | 62.0±0.1 | 54.6±0.1 | 44.9±0.1 |
| m               | 2.47±0.01 | 2.47±0.10 | 2*     | 2*     |

* indicates a fixed parameter
We next examined how the differences among network structures affected the chitosan hydrogel moduli, macromolecular transport through hydrogel pores, and enzymatic degradation of the hydrogels, all of which are critical aspects for applications in tissue engineering and therapeutic agent delivery. In addition to the structural characteristics, such properties are largely affected by the polymer fraction of the hydrogel. Therefore, we match the polymer fractions of the hydrogels at swelling equilibrium as can be seen in Figure 3A.

We measured the storage modulus, \(G'\), of each chitosan hydrogel using an oscillatory shear rheometer. The results of the measurements are shown in Figure 3B. \(G'\) is considered equivalent to the shear modulus, provided the loss modulus is negligible. This was the case for all chitosan hydrogels investigated in this study (Figure S1). We expected \(G'\) of SP would be highest among the four chitosan hydrogels. This was due in part to its structural homogeneity, its average DP that is inversely proportional to the number of crosslinks being smallest, and its small proportion of unreacted methacrylate groups (Figure 2A). However, \(G'\) of SP was found to be the lowest among the hydrogels at 7 kPa. The shear modulus of SP estimated by the phantom network model of classical rubber elasticity is 144 kPa, and we assumed a functionality of four at crosslinks in SP. Such overestimation typically arises when topological defects, such as dangling chains and loops, are ignored. We thus inferred that a large proportion of crosslinking in SP probably occurred by intra-chain cyclization, which formed elastically inactive loops.

In light of these results, we expected that \(G'\) of \(RP_{CT}\) would be higher than that of SP. This is because the probability of crosslink formation by methacrylate groups from at least two different chitosan chains, which made the crosslink elastically active, significantly increased with the average DP. As expected, the measured \(G'\) of \(RP_{CT}\) was higher than that of SP. However, even though the average DPs of \(RP_L\) and \(RP_H\) were significantly higher than that of \(RP_{CT}\), we found that \(G'\)s of \(RP_L\) and \(RP_H\) were lower than \(G'\) of \(RP_{CT}\). This can be understood through the fact that the total number of crosslinks in each hydrogel is inversely proportional to the average DP. From a comparison of two competing effects resulting from higher DP in \(RP_L\) and \(RP_H\) relative to \(RP_{CT}\), i.e., fewer crosslinks but a higher probability of the crosslinks being elastically active, we concluded the impact of the former was greater for lower \(G'\)s of \(RP_L\) and \(RP_H\). This was understandable, assuming the average DP of \(RP_{CT}\) was sufficiently high for most crosslinks in \(RP_{CT}\) to be elastically active.

Next, we investigated how hydrogel network structure affected transport of macromolecules through pores. We used fluorescently-labeled dextran as a model macromolecule due to its wide range of Stokes diameters with a narrow size distribution. We chose a dextran of 4 kDa, which had a Stokes diameter of 2.8 nm that was similar to that of the lysozyme (3.8 nm). We found that the diffusivity of 4 kDa dextran (\(D_{4k}\)) increased with decreasing average DP. In accordance with \(G'\) of \(RP_{CT}\) being greater than \(G'\) of SP, we found that \(D_{4k}\) in \(RP_{CT}\) was less than \(D_{4k}\) in SP, which again suggested that a large proportion of the crosslinks in SP were elastically inactive. We expected that local \(D_{4k}\) values within chain bundles in \(RP_L\) and \(RP_H\) characterized by densely branched network structures would be significantly reduced, such that macromolecular transport within \(RP_L\) and \(RP_H\) would most likely occur through low-density regions containing weakly bound chains. However, apparent diffusivity is proportional to the volume fraction of pores through which macromolecules are transported and inversely proportional to tortuosity, which are smaller than and greater than one, respectively, for the...
heterogeneous network structures. As a result, measured values of $D_{\text{L}}$ in $RP_L$ and $RP_H$ were less than $D_{\text{L}}$ in $RP_{CT}$ and $SP$, which possessed more homogeneous network structures. An even lower $D_{\text{L}}$ in $RP_L$ could possibly be explained by an additional phase separation occurring at a scale of a few micrometers, as indicated by the laser scanning confocal microscope (LSCM) image and slight opaqueness in the photograph of $RP_L$ (Figure S2).

Finally, we examined degradation of chitosan hydrogels immersed in a lysozyme solution at 37 °C. We first measured temporal changes in thickness, a physical dimension, of hydrogel films attached to a coverslip on one side and exposed to the lysozyme solution on the opposite side. Figure 4A shows the temporal variation of hydrogel thickness normalized by the initial thickness prior to incubation. The film thickness of $SP$ and $RP_{CT}$ initially decreased slightly, then increased suddenly after a few days. Film thickness then rapidly decreased, and complete degradation eventually occurred. With $RP_L$ and $RP_H$, film thickness decreased slightly until Day 2 or Day 3, which was followed by an even more gradual decrease through Day 20. The increase in film thickness observed in $SP$ and $RP_{CT}$ indicated further swelling of the hydrogels, which is associated with cleavage of crosslinks. Crosslinks with a higher DP are comprised of more chitosan chains, so they require more time to be cleaved completely. Therefore, hydrogels with higher average DP values were expected to take longer to exhibit an increase in film thickness by the loss of crosslinks. This is indicated in Figure 4A, which shows time to increased film thickness in the order $RP_L$, $RP_H$ (not observed until Day 20) > $RP_{CT}$ > $SP$.

To better understand the varying degradation behaviors of the four hydrogels shown in Figure 4A, we determined the number of remaining chitosan chains and chain ends generated by lysozyme activity after a given incubation time. For this purpose, we prepared rhodamine-labeled hydrogels to quantify remaining chitosan chains and non-fluorescent but otherwise identical hydrogels. The latter was mixed with thiobarbituric acid (TBA), which reacts specifically with cleaved sites at the reducing ends of the chitosan chains to yield fluorescent conjugates (Figure 4B). Figure 4C shows the fluorescence intensities of rhodamine and conjugated thiobarbituric acid averaged over an entire hydrogel volume and normalized by their respective intensities prior to incubation (Day 0). These were determined using Equation 2.

$$
\hat{I}_{cc(ce)}(t) = \frac{\frac{1}{2} \sum_{i=1}^{p} I_{cc(ce)}(i,t)}{\frac{1}{2} \sum_{i=1}^{p} I_{cc(ce)}(i,t_0)}
$$

where $I_{cc(ce)}(i,t)$ was the average fluorescence intensity of the chitosan chain (cc) or chain ends (ce) measured by LSCM after an incubation time of $t$ at the $i^{th}$ stack along the direction of thickness, which was the direction of net diffusion of the lysozyme. The values $p$ and $p_0$ represented the numbers of stacks used for the LSCM measurements at $t$ and $t_0$ (Day 0). We noted that the error bars representing the standard deviations of $I_{cc(ce)}$ at varying locations ($i^{th}$ stack) along the direction of thickness were relatively small, which indicated that degradation behavior was not highly sensitive along the direction of lysozyme diffusion.
On Day 1 in SP, $i_{cc}$ remained almost unchanged, while $i_{ce}$ increased significantly with a reduction in film thickness. This suggested that degradation of SP occurred mainly at the surface. Here, we distinguish the term degradation from cleavage; in this instance, the former indicates complete removal of chain mass from the hydrogel due to cleavages of, in most cases, multiple chains. By Day 3, $i_{cc}$ had decreased substantially with further swelling, indicating that degradation occurred throughout the hydrogel volume with the loss of crosslinks. In $RP_{CT}$, $i_{cc}$ remained almost unchanged along with a steady decrease in film thickness until Day 5, which indicated similarity to SP in that degradation occurred mainly at the surface. We found that lysozyme partitioning in $RP_{CT}$ was substantially greater at the surface until Day 5, as shown in Figure 4D. However, we noted that $RP_{CT}$ exhibited a higher resistance to bulk degradation than SP. This was thought to be due in part to smaller elastically effective pores as indicated by a smaller correlation length and a lower $Da_k$ (Figure 3C); however, the greater DP of crosslinks was presumed to be a more important factor. Additional degradation experiments were performed with confined hydrogel films. Coverslips were chemically attached to both the top and bottom surfaces of the hydrogel films, such that further swelling along the direction of lysozyme diffusion was restricted. The results supported our conclusion that degradation of $RP_{CT}$ was mostly limited to the surface until Day 5 (Figure S4).

However, we observed marked differences in the degradation behaviors of $RP_{L}$ and $RP_{H}$. We expected that lysozyme partitioning within a densely branched network structure of chain bundles would be largely limited, such that initial degradation of $RP_{L}$ and $RP_{H}$ would occur predominantly in low-density regions containing weakly bound chitosan chains. In this case, chain bundles would be degraded slowly by cleavage of chitosan chains located at the periphery of the chain bundles. Such degradation characteristics in $RP_{H}$ were evidenced by a steady decrease of $i_{cc}$ until Day 7 and a significant increase of $i_{ce}$ at Day 3. Film thickness was only slightly reduced between Day 0 and Day 3 and decreased little through Day 20. In addition, although $Da_k$ was lower in $RP_{H}$ than it was in $RP_{CT}$, we observed nearly equilibrium partitioning of the lysozyme in $RP_{H}$ at Day 5 (Figure 4D). This was in contrast to $RP_{CT}$, which implied that the lysozyme migrated rapidly throughout the hydrogel volume due to early-stage bulk degradation of the low-density regions. We therefore concluded that $RP_{H}$ was skeletonized by chain bundles, which allowed $RP_{H}$ to resist bulk disruption for an extended period.

We observed similar degradation behavior in $RP_{L}$ with the exception of a smaller increase of $i_{ce}$ at Day 3. This
could be explained by formation of more crosslinks in low-density regions of $RP_L$, due to prolonged, though slower, initiation during its formation. Therefore, we expected that low-density regions would degrade more slowly in $RP_L$ than in $RP_H$, which was also indicated by a delay in the equilibrium partitioning of the lysozyme in $RP_L$ (Figure S3) compared to $RP_H$. In determining the concentration of remaining chitosan chains, measurements made by LCSM ($RP_H > RP_L$ in Figure 4C) and epi-fluorescent microscopy ($RP_H < RP_L$ in Figure S4) yielded contradictory results, although the differences were not significant. Proposed mechanisms for the degradation of $RP_L$ and $RP_H$ are illustrated in Figure 5.

![Figure 5](image)

*Figure 5. A schematic illustration of the heterogeneous network structures of $RP_L$ and $RP_H$ with proposed mechanisms for enzymatic degradation of (A) low-density and weakly bound regions and (B) chain bundles.*

In conclusion, chitosan hydrogels were made from methacrylate-functionalized macromers using various crosslinking methods, which affected the resulting average DP and heterogeneity of network structures in the hydrogels. Variation of crosslinking methods resulted in marked differences, particularly in lysozyme degradation behavior, as well as in shear moduli and macromolecular transport. To enhance their utilization in tissue engineering applications or drug delivery systems, further investigation of these hydrogels is needed. For examples, additional input variables, such as polymer fractions, the types and concentrations of chain-transfer agents, molecular weights of macromers, or degree of methacrylate modification, should be included. Other relevant properties, including the phenotypes of embedded cells and release profiles of encapsulated therapeutic agents, should also be considered.

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Supporting Information

Effects of crosslinking methods on network structure and enzymatic degradation of methacrylate-functionalized chitosan hydrogel

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Experiments

Materials Chitosan (CS, Mn = 48 kDa and a degree of deacetylation = 90 mol%) was purchased from Golden-Shell Pharmaceutical (Zhejiang, China). Glycidyl methacrylate (GMA), DL-dithiothreitol (DTT), 2-hydroxy-2-methylpropiophenone (Darocur 1773), acetic anhydride, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), 3-(trichlorosilyl)propyl methacrylate, rhodamine B isothiocyanate (RBITC), dimethyl sulfoxide (DMSO), ethanolamine, human lysozyme, and thiobarbituric acid (TBA) were purchased from Sigma Aldrich (Yongin, Korea). Fluorescein labelled dextran (FITC-dextran) was purchased from TdB consultancy AB (Uppsala, Sweden).

Synthesis of methacrylate functionalized chitosan macromer (CS-GMA) CS-GMA was synthesized as described in the previous literature1. Briefly, a 100 mg of chitosan was dissolved in a 100 ml of 0.4 M acetic acid solution, and a 500 μL KOH (50 mM) solution and 498 μL of GMA (6 times the total moles of a monomeric unit of a chitosan chain) were added to the solution. After the reaction proceeded for 24 hours at 60 °C, a 70 μL of acetic anhydride was added to the reaction mixture for an N-acetylation modification, which is known to affect the degradation kinetics as well as solubility of CS2-3. Upon the completion of reaction, the solution was dialyzed against 0.1 mM hydrochloric acid solution (pH 4) for three days and dried by lyophilization. The degrees of methacrylate substitution and N-acetylation were measured by 1H-NMR (500 MHz, Bruker) to be 18.2 mol% and 56.0 mol%, respectively.

Synthesis of chitosan hydrogel using CS-GMA Chitosan hydrogels were synthesized by either chain-growth or step-growth polymerizations. For a free radical initiated chain-growth polymerization, the pregel solution is prepared by dissolving a 5 mg of CS-GMA and 0.06 μL of a photo-initiator, 2-hydroxy-2-methylpropiophenone, in a 20 μL of deionized water to make a 25 w/v% of CS-GMA and a 0.3 v/v% of photo-initiator, respectively. The pregel solution was then injected into the capillary channel made of two coverslips, one of which is pre-functionalized by a 0.4 v/v% ethanol solution of 3-(trichlorosilyl)propyl methacrylate for a covalent attachment to hydrogels, and two 140 μm-thick spacers. The capillary channel filled with the pregel solution was irradiated by a UV LED (365 nm) with a varying power density, 13.4 mW/cm² (Mightex, PLS-0365-030-11-S Type B) or 268 mW/cm² (LIGHTNINGCURE UV-LED, Hamamatsu Photonics) but with an identical dose (0.8 J/cm²).

For a free radical initiated chain-growth polymerization in the presence of a chain-transfer agent DTT, the same pregel solution was used except that a 0.33 mg of DTT was added to the solution such that the molar ratio of
methacrylate groups in CS-GMA to thiol groups of DTT was 1:1. A weight ratio of CS-GMA to DTT was 15:1. The pregel solution was loaded into the same capillary channel and was irradiated by UV LED with a power density of 268 mW/cm². For a step-growth polymerization, the same pregel solution containing a 0.33 mg of DTT but without the photo-initiator was used except that 0.3 μL of DBU (0.3 v/v%) was added as a catalyst for a thiol-ene reaction. The pregel solution was loaded into the same capillary channel and left for 4 hours to complete the reaction. Upon a completion of hydrogel formation, all the hydrogels were thoroughly washed with deionized water.

**Measurements of the polymer fraction of hydrogel at an equilibrium swelling** The polymer fraction of hydrogel at an equilibrium swelling was calculated as \( W_{\text{dry}}/W_{\text{wet}} \), where \( W_{\text{dry}} \) and \( W_{\text{wet}} \) represent the weight of dried chitosan polymer and the weight of hydrogels at an equilibrium swelling, respectively.

**Measurements of hydrogel mechanical properties** Free-standing 500 μm-thick chitosan hydrogel films were fabricated and cut into 8 mm-diameter disks using a biopsy punch. A storage and loss modulus of each chitosan hydrogel was measured using a parallel plate rheometer (DHR-3, TA instruments). A normal compressive stress of 10 mN was applied to the sample to avoid a slippage of the sample from plates during the measurement. The temperature of plates was maintained at 25 °C. Frequency sweeps were carried out over a range from 0.5 rad/s to 100.0 rad/s at a fixed strain of 0.4 %.

**Raman spectroscopy** Chitosan hydrogels were dried prior to the Raman spectroscopic measurement to obtain a stronger signal. The Raman spectrum of each dried chitosan network was obtained using the Raman confocal microscope (a 785 nm laser and 20x objective, RAMAN force, Nanophoton) over a range from 307 cm⁻¹ to 2300 cm⁻¹. Each spectrum was corrected by subtracting a background spectrum and adjusted to match the scattering intensity of a reference peak at 1654 cm⁻¹, which corresponds to the amide in N-acetylation groups⁴.

**Small angle X-ray scattering (SAXS)** The X-ray scattering analysis was conducted using a SAXS instrument (NANOPIX, Rigaku Co., Japan) installed at HANARO, KAERI. Scattering patterns were obtained in the range of 0.003 Å⁻¹ < q < 0.18 Å⁻¹ \((q = 4\pi \sin(\theta/2)/\lambda, \text{where } q \text{ and } \theta \text{ are the scattering vector and the scattering angle, respectively})\) with X-rays of 1.54 Å of Cu Kα radiation, which is generated from a rotating anode generator operated at 40 kV and 30 mA. The 500 μm-thick rectangular samples were loaded on the stage with the windows of Kapton films. The measured scattering profiles were corrected by a subtraction of the empty cell scattering intensity. The 1D circular averaged scattering patterns were analyzed using the analysis package which was distributed by the National Institute of Standards and Technology (NIST)⁵.

**Measurements of FITC-dextran diffusion in chitosan hydrogels** Diffusion of FITC-dextran with varying molecular weights, 4 kDa, inside chitosan hydrogels was monitored using an epi-fluorescence microscope (Axio Observer A1, Zeiss, 10x objective lens). Chitosan hydrogels were prepared inside the capillary channel made of two coverslips, both of which are pre-functionalized by 3-(trichlorosilyl)propyl methacrylate, and two 140 μm-thick spacers such that resulting hydrogels are exposed to surrounding medium only through side walls of 140 μm-thick. As-prepared chitosan hydrogels sandwiched by coverslips were immersed in deionized water for several days to completely extract unbound species. Each chitosan hydrogel still sandwiched by coverslips and gently blown by nitrogen gas to remove surrounding water was then located in the glass dish placed on the microscope stage. Next, an aqueous solution of 0.3 mg/ml FITC-dextran of 4 kDa was filled into the glass dish, and the fluorescent intensity inside the hydrogel was measured by the microscope at a diffusion time of 30 min, 1 hour, 2 hours, and 4 hours. A mild, continuous flow of the FITC-dextran solution was generated inside the glass dish to ensure a constant FITC-dextran concentration at the side wall of hydrogels.

Diffusivity of FITC-dextran inside the hydrogel \( D_w \) was estimated by fitting the measured fluorescent intensity \( I(x, t) \) to the analytical solution of an one-dimensional, transient continuity equation with a constant FITC-dextran concentration at one end \((x = 0)\) and a semi-infinite condition at the other end.

\[
\frac{I(x,t)}{I_0} = K \cdot \text{erfc} \left( \frac{x}{4\sqrt{D_w}t} \right)
\]  

(1)
where $I_o$ and $K$ are the fluorescent intensity of FITC-dextran outside the hydrogel and the equilibrium partition coefficient, respectively, where the latter is also a fit parameter.

**Measurements of enzymatic degradation of chitosan hydrogels by lysozyme** Enzymatic degradation of chitosan hydrogels by lysozyme was monitored by the laser scanning confocal microscope (LSCM, Zeiss LSM 710, 10x and 20x objective lenses). Degradation was characterized by three independent modes; a change of hydrogel thickness, a change of polymer concentration, and a change of chitosan reducing-end concentration. For measuring changes of hydrogel thickness and polymer concentration, fluorescently labelled chitosan hydrogels were used. First, the CS-GMA solution was mixed with a 20 μL DMSO solution of RBITC (1.5 w/v%) for 3 hours, dialyzed against deionized water, and lyophilized. Next, RBITC labelled CS-GMA solution was loaded into the capillary channel with one coverslip pre-functionalized with 3-(trichlorosilyl)propyl methacrylate, and RBITC labelled chitosan hydrogels were formed by a UV initiated chain-growth polymerization or a base-catalyzed step-growth polymerization. Upon a complete removal of unbound species, each chitosan hydrogel attached to a coverslip was immersed in a 0.07 mM aqueous solution of lysozyme (10 mM of phosphate buffered saline, pH = 7.4) maintained at 37 °C for varying amounts of times. After a given period of time, each chitosan hydrogel was withdrawn, and the hydrogel thickness and polymer concentration were measured by LSCM. The lysozyme solution was refreshed daily.

For measuring a temporal change of chitosan reducing-end concentration, chitosan hydrogels made of identical methods but using non-fluorescent CS-GMA. Each chitosan hydrogel was incubated in a 0.07 mM aqueous solution of lysozyme maintained at 37 °C for a given period of time, withdrawn, washed with deionized water, and immersed in a 10 ml of aqueous solution of TBA (1.8 wt%, pH = 3) maintained at 80 °C for 1 hr. Upon a completion of the reaction, chitosan hydrogels were thoroughly washed with deionized water, and the intensity of chromophore produced by the reaction of chitosan reducing-end with TBA (532/553 nm), which is an indication of the chitosan reducing-end concentration, was measured by LCSM. We confirmed that TBA did not react with hydroxyl or amine groups presented in chitosan chains as a mixing of TBA with an excess amount of ethanolamine at the identical reaction condition did not produce any fluorescence. A fluorescent intensity of TBA-conjugated hydrogel at each time of incubation were obtained using an independent hydrogel sample of identical crosslinking method.
**Figure S1.** A loss modulus $G''$ of each chitosan hydrogel measured by the oscillatory shear rheometer. Error bars indicate standard deviations from independent measurement of three to seven samples for each hydrogel.

**Figure S2.** (A) A LSCM image of $RP_L$ showing a spatial variation of chain concentration at an average length scale of 3 – 4 μm. (B) Photographs of each hydrogel placed over printed characters, which indirectly indicates opacity of the hydrogel.

**Figure S3.** A fluorescent intensity of lysozyme along the direction normal to the exposed surface of confined hydrogel films of (A) $RP_L$ and (B) $SP$. Each fluorescent intensity at a given incubation time is normalized by the fluorescent intensity of the lysozyme solution located outside the hydrogel.
Figure S4. A fluorescent intensity of remaining chitosan chains along the direction normal to the exposed surface of confined, rhodamine-labelled hydrogel films of (A) RP_L, (B) RP_H, (C) RP_CT, and (D) SP. Each fluorescent intensity at a given incubation time is normalized by the one measured at Day 0 (indicated as a horizontal dashed line). A position of the surface of each hydrogel at Day 0 is also indicated as a vertical dashed line.

References


