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Injectable in situ Physically and Chemically Crosslinkable Gellan Hydrogel

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Abstract

An injectable, in situ physically and chemically crosslinkable gellan hydrogel is synthesized via gellan thiolation. The thiolation does not alter the gellan's unique 3-D conformation, but leads to a lower phase transition temperature under physiological conditions and stable chemical crosslinking. The synthesis and hydrogels are characterized by ¹H NMR, FT-IR, CD, or rheology measurements. The injectability and the tissue culture cell viability is also tested. The thiolated gellan hydrogel exhibits merits, such as ease for injection, quick gelation, lower gelling temperature, stable structure, and nontoxicity, which make it promising in biomedicine and bioengineering as an injectable hydrogel.

1. Introduction

Hydrogels are crosslinking polymer networks that contain large amounts of water. Their biophysical similarity to soft biological tissues makes them useful to the biomaterials research community.¹ Injectable hydrogels are hydrogels capable of gelling in situ due to chemical and/or physical crosslinking development after injection. Because of the advantages over their conventional counterparts, injectable hydrogels have found a great

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number of applications in tissue engineering, drug delivery, cell and protein transplantation.^{2–5} The most attractive advantages are that they are easily deliverable by minimally invasive techniques, and are capable of filling native or potential cavities by conforming to different shapes, which may be difficult to prefabricate.⁶

Gellan gum, hereafter referred to as gellan, is a microbial polysaccharide biopolymer produced by the bacterium *Pseudomonas elodea*.⁷ The chemical structure of the deacylated gellan consists of a repeating unit of tetrasacchride (D-glucose, D-glucuronic acid, and L-rhamnose in a molar ratio of 2:1:1)⁸ (Scheme 1). Upon temperature decrease, gellan molecules undergo a rapid random-coil to double-helix phase transition, and when cations (e.g., Na⁺, K⁺, Ca²⁺, Mg²⁺) are present, a physical hydrogel forms. The mechanism of gelation involves the formation of double-helical junction zones followed by aggregation of the double-helical segments, resulting in a 3-D network via complexation with cations and hydrogen bonding with water.⁸ It has been confirmed that the carboxylic groups in glucuronic acid serve as the ionic complexation sites binding gellan chains to each other via cations.⁹ Divalent cations (e.g., Ca²⁺, Mg²⁺) exhibit much stronger interactions with gellan chains than monovalent ones (e.g., Na⁺, K⁺).⁹ Most recently, gellan-based physical hydrogels have been explored in tissue engineering because of their high biocompatibility, biodegradability, and injectability.^{2, 10–12} However, substantial problems remain before the gellan hydrogels can become genuinely injectable for practical biological use – its gelation temperature is much higher than human body temperature (37 °C)² and it gels in seconds in PBS medium, leaving no window of time for manipulation.¹⁰ In addition, gellan physical hydrogels induced by cations tend to become weaker in physiological conditions due to the exchange of divalent cations by monovalent ones.¹³ To lower the gelation temperature, most recently, chemically scissoring the gellan backbone via oxidation cleavage was employed to reduce their molecular size to optimize the gelation temperature for injection.¹⁴ The as-prepared gellan was able to form physical hydrogels at lower temperatures, but no chemical crosslinking was introduced. On the other hand, Reis et al. introduced photo-crosslinkable double bonds to the gellan chains via gellan methacrylation to provide gellan hydrogels with a stable network in physiological medium.¹⁵ The modified gellan was then chemically crosslinked by UV irradiation. The gellan with limited modification was observed to maintain its ability to gel in PBS; however, the effect of modification on gelation temperature was not reported.

Here, we present a simple way to create an in situ chemical crosslinkable gellan hydrogel with decreased gelation temperature, while keeping its physical gelation capacity. One of the applications of this hydrogel in our research is for use in developing an injectable intraocular lens, which is capable of auto-focusing, functioning like a young human lens owing to the soft property of the hydrogels. Specifically, a thiol group containing amino acid, L-cysteine, has been introduced to the gellan backbone via carbodiimine chemistry. A chemical crosslinking was developed between gellan chains while the thiol groups underwent air oxidation in physiological conditions, giving a stable chemical hydrogel. On the other hand, the minimum modification did not alter gellan's 3-D conformation, but decreased the gellan's phase transition temperature, or its gelation temperature. We propose that the possible mechanism behind the lower gelation temperature is due to the reduced cation binding sites on the gellan backbone and the steric effect of the attached small molecule.

This gellan with decreased gelation temperature exhibited both physical gelling and chemical crosslinking ability, making it desirable for in vivo applications in tissue engineering. Fourier-transform infrared spectroscopy (FT-IR), proton nuclear magnetic resonance (¹H NMR), and Ellman's reagent were used to characterize the modification. Circular dichroism (CD) and rheological measurements were employed to characterize the molecular conformation of the modified gellan, its sol/gel transition, and gelation behaviors. The chemical hydrogel evolution was demonstrated via monitoring its storage modulus development. The effect of PBS and pH on the oxidation rate of thiol groups in air was investigated. The injectability of the thiolated gellan hydrogel was demonstrated in vitro near body temperature using a regular syringe needle. Cell viability was tested using Mitochondrial Apoptosis Detection Kit on ARPE-19 cells.

2. Experimental Section

2.1. Materials

Deacetylated gellan gum ($\overline{M}_w = 1000$ kDa), L-cysteine hydrochloride monohydrate, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), Dulbecco's phosphate-buffered saline (PBS), and all tissue culture reagents, including Dulbecco's modified Eagle's/nutrient mixture F-12 Ham's medium (DME/F-12), and fetal calf serum (FCS) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as received. Retinal pigment epithelial cells (ARPE-19) were purchased from American Type Culture Collection (Manassas, VA, USA). A Mitochondrial Apoptosis Detection Kit (JC-1) was obtained from Genscript, Piscataway, NJ, USA.

2.2. Synthesis of Thiolated Gellan

Thiolated gellan was synthesized at room temperature (20 °C) using a modified method.¹⁶ Typically, 0.90 g EDAC was added to 30 mL 1% (w/v) deacetylated gellan solution at room temperature. The reaction was allowed to proceed for about 1 h. 0.90 g L-cysteine hydrochloride was then added and the pH was adjusted to 5.0 using 1 M NaOH. The reaction mixture was incubated for 3 h. The resulting mixture was purified via dialysis (molecular-weight cutoff (MWCO) 12–14 kDa) against HCl acidified (pH ≈ 3), N₂ saturated DI water at ≈5 °C, until an absorbance at 412 nm, due to the presence of thiol groups in the dialysis medium, was undetectable using Ellman's reagent DTNB.¹⁷ A control experiment was carried out following the same procedures except EDAC was omitted. The purified sample was dried by lyophilizing to give a white, fibrous solid, thiolated gellan. The dried sample was stored at ≈4 °C under N₂ for further use.

2.3. Characterization of Thiolated Gellan

The gellan thiolation was assessed by ¹H NMR spectroscopy and FT-IR spectroscopy. ¹H NMR spectra were obtained on a Varian Mercury-300 MHz spectrometer. Samples were prepared at 0.5% (w/v) in D₂O. FT-IR spectra were recorded on a Nicolet Nexus 470 FT-IR Spectrometer. The thin film samples were obtained by casting polymer solutions onto flat glass surfaces and dried under vacuum. Circular dichroism (CD) was performed on a Jasco Model J-715 Spectropolarimeter in the range of 250–190 nm with a scanning rate of 20 nm

min⁻¹. The CD spectra were measured during cooling from 40 to 15 °C and maintained within ± 0.1 °C at each point using a RTE bath/circulator with microprocess controller. The thiol group content in the thiolated gellan was determined using Ellman's reagent DTNB.¹⁷ The degree of thiolation (DT, %) was the fraction of modified carboxylic groups over the total carboxylic groups in each gellan molecule.

2.4. Preparation of Thiolated Gellan Hydrogels

0.2, 0.5, and 1.0% (w/v) thiolated gellan solutions were prepared at ≈ 60 °C in DI water and PBS at pH = 7.5. The solutions were transferred into tissue culture dishes (35 $\times$ 10 mm²) before they gelled, and were stored in a humidified incubator at room temperature allowing air flow for oxidation. The gelation point was determined using a simple inversion method: the solution was considered to be gelled if it lost its mobility when the dish was turned vertically and the gelation time was record (n = 3).

2.5. Hydrogel Liquifying and Regelling

The hydrogel is reversible. To test this character, 5 g 0.5% (w/v) thiolated gellan hydrogel was crushed and mixed with 15 mg reducing agent DTT and 2 mL deionized (DI) water. The mixture was stirred overnight at room temperature. A visually clear solution indicated the complete liquefaction of hydrogel. The sample was dialyzed, lyophilized, and regelled following the same method as described above.

2.6. Temperature-Dependent Rheological Measurements

Rheological measurements were carried out on a Vilastic-3 oscillatory tube rheometer. The measuring geometry was a precision cylindrical tube with an inner diameter of 0.991 mm and 64.5 mm long. A thermal scan with 5% constant shear strain and 1.0 Hz constant frequency was used to measure the transition point of the polymers in water and PBS by cooling the samples, equilibrating within ± 0.1 °C at each point. A few milligrams of DTT was added to the samples to prevent disulfide crosslinking during the measurements. All the samples were loaded while their temperature was above the starting temperature of scans.

2.7. Time-Dependent Rheological Measurements

To present the evolution of chemical crosslinking and hydrogel formation of the thiolated gellan in DI water, a time scan was used to monitor the development of the mechanical properties (storage modulus) of 0.5% (w/v) thiolated gellan solution (pH = 7.5) at room temperature. As air had no access to the samples loaded in the tubing during the measurement process, to facilitate the oxidation the rheology chamber was filled with freshly oxygen-saturated water and the pH was adjust to 7.5.

2.8. Thiol Group Oxidation

To evaluate the effect of PBS and pH on the air oxidation rate of the thiol groups in the thiolated gellan, the content of thiol groups was monitored on 0.2% (w/v) thiolated gellan in water and PBS media with pH = 7.5, 8.5, and 9.5 at room temperature. The samples were stored in a humidified incubator. At determined intervals, aliquots were removed and the content of thiol groups in the samples was determined using Ellman's reagent as mentioned

previously. All experiments were carried out in triplicate and the results were averaged with arithmetic mean $\pm$ SD.

2.9. Injectability Test

To study the injectability of the thiolated gellan hydrogel, 0.5% (w/v) thiolated gellan solution (PBS, pH = 7.5) was loaded into a 10 mL syringe at $\approx 40^\circ\text{C}$ and injected directly into a poly(methyl methacrylate) (PMMA) mold consisting of 5 spherical cavities (10 mm diameter) via a syringe needle ($20 \times 11/2$ gauge). The PMMA mold was preheated at 37°C in an oven.

2.10. Cytotoxicity Assay

To test the biocompatibility of the thiolated gellan hydrogel, the thiolated gellan was dissolved in sterile water at pH ≈ 7.4 and mixed with DME (2 $\times$) at 50°C to make a final polymer concentration of 0.15%. 2 mL solution was transferred immediately into 60 mm dishes. ARPE-19 cells were plated concurrently onto 22 mm glass cover slips in DME/F-12 containing 10% FCS and antibiotics at a density of $\approx 50\,000$ cells and allow to attach for 24 h. Cover slips with cells (15–20% confluent) were placed on top of the thiolated gellan hydrogels and 2 mL of DME/F-12 containing 10% FCS and antibiotics was added. An additional 0.5 mL of media was added at day 4 and day 6. Pictures were taken at day 8 and cells were stained for 15 min using the Mitochondrial Apoptosis Detection Kit. This kit is a fluorescent based assay using the dye JC-1 for detecting the loss of mitochondrial transmembrane potential. In healthy cells there is a negative charge established by the intact mitochondrial membrane potential which allows the lipophilic dye bearing a delocalized positive charge to enter and accumulate in the mitochondria. The aggregated form becomes fluorescent red. This mitochondrial potential collapses in apoptotic cells, and the JC-1 remains dispersed in the cytoplasm and hence remains in the green fluorescent monomeric form.

3. Results and Discussion

3.1. Gellan Thiolation and Characterization

To prepare thiolated gellan, the carboxylic acid residues of glucuronic acid on deacylated gellan were activated with EDAC.¹⁴ The reaction of activated carboxylic acid residues with the free amine residues of cysteine resulted in the formation of amide bonds between the gellan chains and cysteine molecules with one water as the leaving molecule (Scheme 1). The carboxylic group in gellan is the functional group that has been most frequently used in the gellan chemical modification. Dentini et al. prepared gellan chemical hydrogels through self-crosslinking of gellan chains in the aqueous physical gel state.¹⁸ Coviello et al. crosslinked gellan chains via L-lysine ethyl ester moieties.¹⁹ In both cases, the carboxylic groups of the gellan were activated using EDAC. This carbodiimide chemistry is also frequently employed in other polysaccharide modification to introduce chemical crosslinking.^{20–22} To prevent the thiolated gellan from oxidation by air during the purification, cysteine was added in excess amount in the reaction mixture and the purification was carried out in an oxygen excluded (through N₂ bubbling) and acidic medium (pH ≈ 3) at low temperature ($\approx 5^\circ\text{C}$). The resulting thiolated gellan was a white,

fibrous solid. Figure 1 shows the ^1H NMR patterns of the unmodified gellan (control) and thiolated gellan obtained at room temperature. The signals appearing around $\delta = 3.2$ and 4.3 represent the protons from the CH_2 groups and the proton connected to the α -carbon in the cysteine. FT-IR spectra (Figure 2) also reveal the existence of the thiol group appearing at 2550 cm^{-1} as a weak peak in thiolated gellan.²³ It can be concluded that the synthesis happened as expected.

As the thiol group is easily oxidized into disulfide, the thiol group content in the thiolated gellan is proportional to the crosslinking degree. The degree of thiolation was $6.8 (\pm 1.8)\%$ ($n = 5$), indicating that about 6.8% carboxylic groups in the gellan backbone were conjugated with cysteine molecules at room temperature. 1% (w/v) unmodified gellan aqueous solution has a random-coil to double-helix transition at $\approx 27^\circ\text{C}$, which was demonstrated by the rheological measurements (Figure 3) and is in good agreement with literature.⁸ In Figure 3(a), both storage modulus G' and loss modulus G'' increased sharply around 27°C , which registered the gellan molecular conformation transition from random-coil to double-helix upon cooling. At room temperature (20°C), 1% (w/v) gellan had significantly increased G' and G'' , indicating it was in the double-helix conformation. It can be inferred that at room temperature the gellan was thiolated when it was in the double-helical state, where the carboxylic acid groups near the ends of the gellan backbone could be more efficiently activated or accessible for conjugation.

To evaluate the effect of thiolation on the gellan phase transition, a rheological measurement was also performed on 1% (w/v) thiolated gellan in the absence of PBS [Figure 3(b)]. Comparing with Figure 3(a), it is found that the thiolated gellan retained its random-coil to double-helix transition characteristic and had almost the same phase transition temperature ($\approx 27^\circ\text{C}$) as that of unmodified gellan. The rheological measurements confirm that the thiolation at a low degree did not alter the 3-D structure of gellan molecular architecture. The thiolated gellan hence still possessed the capability to form instant physical hydrogels under the influence of ions. This physical gelation property is helpful in preventing leakage after hydrogel injection, which is a challenge in applications of injectable hydrogels for tissue engineering.

3.2. Thiolated Gellan Hydrogels

The thiolated gellan was soluble in water without heating when pH was above 6. To prepare hydrogels, a series of thiolated gellan solutions were made with or without PBS at $\text{pH} = 7.5$. At room temperature, the thiolated gellan formed a transparent chemical hydrogel due to the disulfide crosslinking developed from the thiol group air oxidation. The gelation time, t_{gel} , is presented in Table 1.

It is well known that gellan aqueous solutions will not gel without the influence of cations, such as in PBS. The hydrogel formation from thiolated gellan in DI water at low concentration clearly indicates that the hydrogel formation is a result of chemical crosslinking. It is known that gellan physical gels are thermally reversible and soluble in water due to the dilution or partial loss of cations.²⁴ It is noteworthy that the thiolated gellan hydrogels were not only stable when exposed to excess water for prolonged periods, but also exhibited strong thermal stability. For instance, 0.2% thiolated gellan physical hydrogel

retained its gelation status at even over 80 °C, where unmodified gellan hydrogel was liquefied (Figure 4). The water-insolubility and thermal stability of the thiolated hydrogels further confirm the existence of the strong chemical crosslinking in the gellan hydrogel network.

Figure 5 shows the sol/gel transition of the thiolated gellan solutions in PBS. In comparison with gellan, which spontaneously forms physical gels at high temperatures upon cooling in the presence of PBS,²⁵ thiolated gellan showed significantly decreased gelation temperature and increased gelation time. Table 2 summarizes the transition and gelation points and the temperature difference between the transitions. It can be seen that the gellan not only has higher gelation temperatures (45.5, 47.4 and 50.5 °C for 0.2, 0.5, and 1.0% w/v), but also a very narrow transition temperature range (, 1 °C for all the concentrations), which greatly limit the gellan as an injectable hydrogel. The thiolated gellan demonstrated much lower gelation temperatures (20.5, 37.2, and 40.85 °C for 0.2, 0.5, and 1.0% w/v) and broader transition temperature ranges (7, 6, and 4 °C for 0.2, 0.5, and 1.0% w/v), implying that the hydrogel can be fabricated at lower temperatures and with a controllable time. In PBS, the gelation of gellan results from two processes upon cooling: random-coil to double-helix transition and helix-helix aggregation under the influence of cations.^{8, 25} It is possible that the thiolation slowed down the latter process because of the steric effects arising from the introduction of cysteine to the gellan chains. Furthermore, thiolated gellan hydrogels formed in PBS utilize a hybrid crosslinking scheme, in which physical gelation is due to the ion effect and chemical gelation due to the disulfide bond crosslinking. This hydrogel therefore further exhibits its advantages over its parents, such as controllable gelation time and stability. The ability to tailor this gelation process makes thiolated-gellan based hydrogels desirable for biological applications in vivo.

To demonstrate the injectability and in situ gelation process, 0.5% (w/v) thiolated gellan solution in PBS was injected into a prefabricated mold via a regular syringe needle at temperature around 37 °C. The hydrogel lens formed with the predesigned shape within several minutes (Figure 6). As the thiolated gellan solution could be injected in a highly purified form and become a hydrogel in mild conditions, it has promising applications in biological tissue engineering as an injectable gel.

3.3. Circular Dichroism (CD)

Circular dichroism is a proven sensitive probe of conformation changes in gellan, which was first established by Crescenzi and coworkers to evidence the existence of an ordered form of gellan in solutions.^{8, 26} Typical CD spectra obtained for 0.5% unmodified gellan aqueous solution on cooling are shown in Figure 7. The solution CD was measured in the wavelength range of 190–250 nm. The patterns are mainly a result of the $n-\pi^*$ transition of the carboxylic chromophore.²⁶ The peaks around 202 nm in the CD spectra reflect the optically active chemical structure of the glucuronic acid unit in the gellan.²⁶ On decreasing the temperature to below 26 °C, the optically active high-order structure of the gellan chain, which is believed to be a double-helix structure, is formed. The CD spectrum is changed to the superposition of the spectrum for the random coil and that of double helix. The drastic change in molar ellipticity at 202 nm at 26 °C indicates the conformation change of gellan

from random coil to double helix, which coincides well with the reported CD results for unmodified gellan at same concentration.⁸ In addition, the change in molar ellipticity at 202 nm is proportional to the change in the population of the random coil.²⁷ Figure 8 exhibits the CD spectra of 0.5% thiolated gellan hydrogel obtained under the same conditions as those of unmodified gellan. It can be seen that all the spectra correspond to the double-helical structure, which strongly supports the rheology measurement results that the double-helical conformation was retained after gellan thiolation. On the other hand, there is no appreciable change in the molar ellipticity at 202 nm within the measured temperature range, indicating that the conformation transition disappeared and the double helices were thermally stable. We attribute this to the disulfide bond formation between the double helical conformation, which confines the polymer chain movement.

3.4. Thiol Group Air Oxidation

To investigate the influence of PBS and pH on the oxidation rate of thiol group in air, the thiol group content was monitored in 0.2% thiolated gellan solutions under different conditions at room temperature. As indicated in Figure 9, the percentage of thiol groups in the solutions decreased with time due to the air oxidation. The air oxidation rate was greatly facilitated in basic media (pH = 8.5 and 9.5). In addition, the presence of PBS also increased air oxidation rate. In basic medium, the thiol groups form thiolate anions. It is known that thiolate anions are much more reactive to oxygen in air than their protonated counterparts.²⁷ In PBS, 0.2% thiolated gellan solution was able to form a physical gel at room temperature as discussed previously. The molecular mobility of thiolated gellan polymer chains was constrained and the thiol groups from different chain strands were brought closer together, favoring intermolecular crosslinking, or the thiol group oxidation.²⁷ This explains why the oxidation rate in PBS medium is much faster than that in water at the same conditions. In addition, the gelation was also observed in the water media when only approximately half of the thiol groups were oxidized, implying that a chemical network could be set up at a low crosslinking degree.

3.5. Gelation Monitoring

The gelation process of the thiolated gellan was monitored via rheology measurements. Figure 10 shows how the mechanical properties (G' and G'') of 0.5% thiolated gellan aqueous solution changes with time. As described in the experimental section, the rheometer chamber was filled with oxygen saturated water. The oxygen diffused into the sample tube to facilitate the oxidation process. During the first period of 10 h, the sample remained liquid, as the G'' is larger than G' .²⁸ After 10 h, G' increased rapidly to the point where it became larger than G'' at about 12.5 h, indicating the initiation of hydrogel network.²⁸ In the following 20 h, G' kept increasing to ≈ 30 Pa and then reached a plateau, suggesting the formation of a firm gel.

3.6. Hydrogel Liquefying and Regelation

One of the other unique properties of the thiolated gellan hydrogel is that it can be liquefied when disulfide bond (S–S) reducing reagents are present, and re-gelled when the thiol groups are oxidized as demonstrated above. The chemical mechanism behind thiolated hydrogel reduction is based on exchange reactions between disulfide bonds from hydrogels

and thiol groups from reducing reagents.²⁷ DTT is the most frequently used reducing agent.^{27, 29} Basic condition favors the reducing process as only the negatively charged thiolate form is reactive.²⁷ In our experiment, the thiolated gellan hydrogels were reduced by adding excessive DTT (5 equivalents of DTT per disulfide) at pH = 7.5–8.0 under ambient conditions. The excessive amount of DTT was used to prevent the reduced thiol groups from oxidization by air. After reaction, the resulting solutions were transparent and no appreciable gel particles could be identified visually, indicating the completion of the reduction reaction. After dialysis and lyophilizing, the recovered thiolated gellan exhibited the same gelation behavior in water and PBS as its counterparts. The hydrogel liquefaction and regelation validates the mechanism of disulfide bond crosslinking. The liquefying can also be employed in recovery of cells, drugs, proteins, or enzymes that are immobilized in the hydrogels for specific applications.

3.7. Biocompatibility of Thiolated Gellan Hydrogel

The compatibility of the thiolated gellan hydrogels was investigated in vitro (Figure 11). As demonstrated in Figure 11, the thiolated gellan showed no adverse effects on growth of ARPE-19 cells for up to 8 d as compared to the control cells. Moreover, the cells had no morphological alternation when growing in the presence of thiolated gellan hydrogel. This demonstrates that the thiolated gellan hydrogel is cytocompatible. The same results were also observed in other research when the disulfide bonds were introduced to the polymers such as collagen, hyaluronan, etc., to fabricate hydrogels.^{27, 30, 31} The cytocompatibility of the thiolated gellan hydrogel can be attributed to following aspects. Gellan, the backbone of the thiolated gellan hydrogels, has a good compatibility and is a FDA approved food additive. Disulfide bonds, the crosslinker of the hydrogels, are also cytocompatible as they have been widely found in proteins in the extracellular matrix.²⁷ Moreover, in this research the disulfide bonds formed under mild conditions to accomplish the gelation, avoiding the use of toxic chemicals, heat, or UV irradiation, which also benefit the material's biocompatibility.

4. Conclusion

Thiolated gellan was synthesized by conjugating gellan with cysteine, a thiol containing amino acid, at room temperature based on carbodiimide chemistry. The thiolation endowed the gellan hydrogel with a stable chemical crosslinking via disulfide formed through thiol air-oxidation under mild conditions. The introduction of cysteine greatly decreased the gellan gelation temperature in PBS possibly due to the slowing of the helix-helix aggregation under the influence of cations, resulting from the steric effect. On the other hand, the thiolated gellan kept its quick physical gelation property in PBS as the thiolation did not alter gellan's characteristic 3-D conformation. This combination of physical and chemical crosslinking mechanisms enabled the fabrication of injectable hydrogels with stable structure in physiological conditions.

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Figure 1.
¹H NMR spectra of gellan and thiolated gellan (solvent: D₂O).

Figure 2.
FT-IR spectra of gellan (a) and thiolated gellan (b).

Figure 3.

The storage modulus G' and loss modulus G'' of 1% (w/v) (a) gellan and (b) thiolated gellan aqueous solutions (pH = 7.4) without PBS upon cooling from 50–10 $^{\circ}\text{C}$. Both solutions show a sharp increase in G' and G'' (sol/gel transition point) at around 27 $^{\circ}\text{C}$, indicating the thiolation has little effect on the gellan backbone.

Figure 4. Thermal stability of the thiolated gellan (solid line) hydrogels compared with that of 0.2% gellan (dashed line) upon heating. Both hydrogels formed in PBS. Below 80 °C, 0.2% gellan had been liquefied while the thiolated gellan remained solid hydrogel state

Figure 5.

Sol/gel transition of gellan (dashed line) and thiolated gellan (solid line) solutions in PBS at (a) 0.2%, (b) 0.5%, and (c) 1.0% (w/v) upon cooling. Gellan solutions show high transition points (gelation temperatures) and narrow transition temperature ranges, while thiolated gellan solutions displayed decreased transition points and increased transition temperature ranges.

Figure 6. Digital pictures of hydrogel lenses fabricated by injection of 0.5% (w/v) thiolated gellan PBS solution (pH = 7.5) into a prefabricated PMMA mold (the diameter of the cavities is 10 mm) at $\approx 37^{\circ}\text{C}$ using a syringe needle ($20 \times 11/2$ gauge).

Figure 7.

CD spectra of 0.5% (w/v) gellan aqueous solutions at various temperatures. The gellan molecules adopted random coils when temperature was 26 °C and above, while double helices when it was at 15 °C. The CD spectra between 26 and 15 °C are the superposition of the spectrum for the random coil and that of double helix.

Figure 8. CD spectra of 0.1% (w/v) thiolated gellan hydrogel at various temperatures, demonstrating a double helical conformation at the measured temperature range (40–15 °C).

Figure 9.

The influence of pH and PBS on the oxidation of thiol groups in 0.2% (w/v) thiolated gellan solutions with (solid curves) and without (dashed curves) PBS at room temperature. The arrows indicate the gelation points.

Figure 10.

The evolution of gelation process monitored via rheological measurements of a 0.5% thiolated gellan aqueous solution without PBS at room temperature. The DI water in the sample chamber was oxygen-saturated to facilitate the oxidation. Inset: a hydrogel slip withdrawn from sample tube after the measurements.

Figure 11.

Digital photos of ARPE-19 cells cultured after 8 d on glass cover slips in the presence of 0.15% thiolated gellan hydrogel. A) Control cells (unstained); B) Control cells stained with JC-1 mitochondrial apoptosis detection kit; C) Cells (unstained) grown in the presence of 0.15% thiolated gellan hydrogel; D) Cells stained with JC-1 mitochondrial apoptosis detection kit grown in the presence of 0.15% thiolated gellan hydrogel.

Scheme 1.

Deacylated gellan structure and its thiolation reactions

Table 1

Gelation time of the thiolated gellan in water, pH = 7.5.

Concentration [% w/v]	t_{gel} [h]
0.2	32 ± 4
0.5	14 ± 2
1.0	4 ± 2

Sol/gel transition and gelling temperature of gellan and thiolated gellan in PBS, pH = 7.5.

Table 2

Concentration [% w/v]	Gellan			Thiolated gellan			
	Start [°C]	End [°C]	[°C]	Gel [°C]	Start [°C]	End [°C]	Gel [°C]
0.2	46	45	1	45.5 ± 0.1	27	20	7
0.5	48	47	1	47.4 ± 0.2	40	34	6
1.0	51	50	1	50.5 ± 0.3	44	40	4
							20.5 ± 2.1
							37.2 ± 1.5
							40.8 ± 0.4