Synthesis and characterization of a novel biocompatible, non-toxic, and fast-gelling chitosan-PEG hydrogel based on Michael addition

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Abstract: This article describes the synthesis and characterization of a novel hydrogel formed through maleimide-thiol conjugation. The hydrogel is produced by combining two precursors: chitosan functionalized with thiol groups and polyethylene glycol (PEG) functionalized with maleimide groups, both of which were analyzed using NMR. The hydrogel was then subjected to several tests to evaluate its properties, including gelation time, swelling behavior, viscoelasticity, and degradation rate. The results showed that the gelation time of the hydrogel could be adjusted by changing the weight percentage of the precursors, as evidenced by the gelation time test. Additionally, the optimal formula for gelation was determined based on references and was used in subsequent experiments. The hydrogel demonstrated excellent flexibility in the swelling study, and the degradation test confirmed that it was biodegradable. The viscoelastic test revealed that the hydrogel was an elastic solid. Overall, the results suggest that this novel hydrogel has potential for biomedical applications.

Keywords: Hydrogel, Chitosan, PEG, Synthesis, Characterization

1. Introduction

Chitosan is a natural biopolymer that has garnered significant interest in the biomedical field due to its biocompatibility, biodegradability, and non-toxicity.[1] It has been explored for various applications, such as drug delivery, wound healing, tissue engineering, and gene therapy.[2] One of the main advantages of chitosan is its ability to form a gel-like substance in acidic conditions. This property has made it an attractive candidate for wound healing applications, as chitosan-based dressings can help prevent infection and promote tissue regeneration.[3] Additionally, chitosan has been used as a scaffold material for tissue engineering, as it can support cell growth and proliferation.[4] Chitosan has also been investigated as a drug delivery system. Due to its positive charge, it can interact with negatively charged drugs and protect them from degradation in the body.[5] It has been used to deliver a wide range of drugs, including antibiotics, anti-cancer drugs, and proteins. Another promising application of chitosan is in gene therapy. Chitosan can form complexes with DNA and facilitate gene delivery to cells. This has the potential to revolutionize the treatment of genetic disorders and other diseases.[6]

Compared with other kinds of hydrogels based on other materials including polyvinyl acetate, tripolyphosphate, sulfobetaine, chitosan hydrogels have more disadvantages. [7-11] Chitosan hydrogel is a natural biopolymer-based material that has gained significant attention in recent years due to its unique properties and wide-ranging applications.[12] Chitosan hydrogel has a unique ability to absorb large amounts of water, which makes it an excellent material for wound healing and tissue engineering applications. When the hydrogel comes in contact with a wound, it forms a protective layer that prevents bacterial

infections and promotes faster healing.[13] Additionally, chitosan hydrogel has been shown to have anti-inflammatory properties that help reduce pain and swelling.[14] In addition to wound healing and tissue engineering, chitosan hydrogel has also been explored for use in drug delivery systems, where it can be used to encapsulate drugs and release them in a controlled manner.[14] This property makes it an ideal material for developing drug delivery systems for a variety of diseases, including cancer. Overall, chitosan hydrogel is a versatile and promising material with a wide range of potential applications in biomedical and pharmaceutical fields.[15] Its unique properties make it an attractive option for researchers and clinicians looking to develop innovative solutions for various medical problems.

However, chitosan hydrogels still have many defects including poor solubility, susceptible to degradation by enzymes, induce an immune response in some individuals, toxicity to the cells, long gelation time with moderate weight percentage hydrogel precursors, etc.[16-20] For instance, Zeng etc., synthesized a chitosan-PEG hydrogel using UV lamp as initiator. However, the light could be harmful to the cells in the biomedical application. Also, their gelation time is much long than 1 hour if they only use 4% chitosan hydrogel precursors.[21]

In this paper, we solved above two defects: long gelation time with moderate weight percentage hydrogel precursors and toxicity from the initiator. We created a hydrogel based on chitosan and 4-arm PEG using maleimide-thiol coupling. We also investigated the hydrogel regarding its gelation speed, swelling behavior, viscoelastic property, and degradation rate.

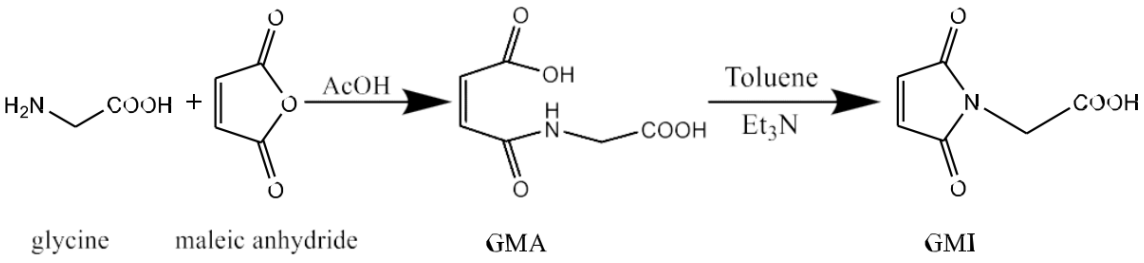
2. Experimental Section

2.1. Materials

VWR was the source of glycine, maleic anhydride, glacial acetic acid, toluene, triethylamine, ethyl acetate, N,N'-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), p- toluenesulfonic acid monohydrate (PTSA), and anhydrous dimethyl sulfoxide (DMSO). Sigma-Aldrich supplied magnesium sulfate, hydrochloric acid (HCl), and ethanol. To synthesize 4-(Dimethylamino)pyridinium 4-toluenesulfonate (DPTS), DMAP and PTSA were used. A Millipore purification apparatus was used to filter deionized water for all experiments.

2.2. Synthesis

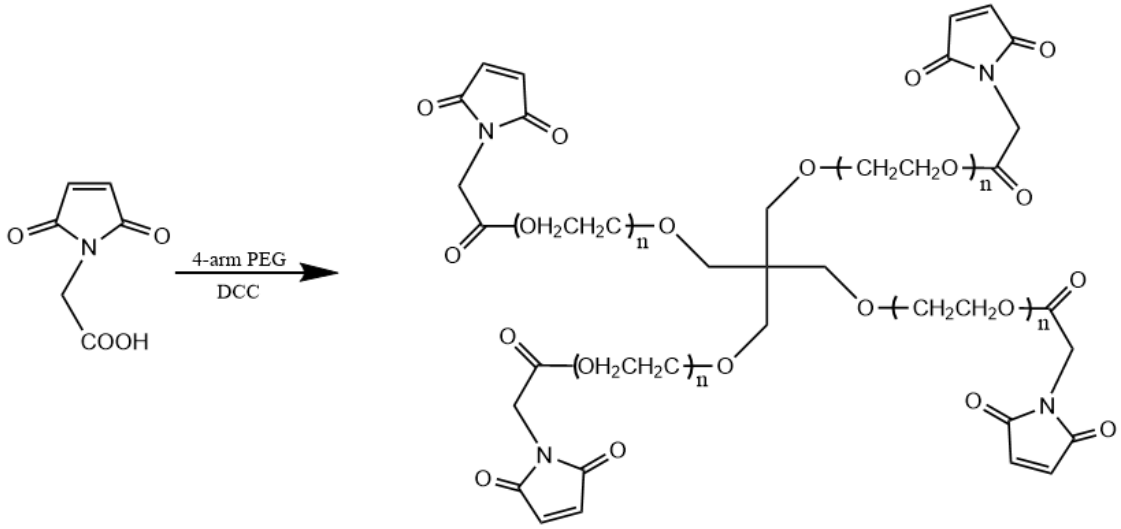
2.2.1. Synthesis of carboxyl end group maleimide (GMI)



Scheme 1. Synthetic route of GMI.

Scheme 1 illustrates the process for preparing GMI. The synthesis of GMI followed the previously described method. In general, a mixture of maleic anhydride (0.1 mol, 9.8 g) in 50 mL of glacial acetic acid was combined with a solution of glycine (0.1 mol, 7.5 g) in 100 mL of glacial acetic acid. The mixture was stirred at room temperature and then filtered. The resulting precipitate was washed with cold water and dried under vacuum. Next, a suspension of N-glycinyl maleamic acid (16.8 mmol, 2.91 g) and Et_3N (35.1 mmol, 3.55 g) in 500 mL of toluene was vigorously stirred and refluxed for 1.5 hours. During this time, a Dean-Stark apparatus was used to remove the byproduct water. The toluene solution was separated from the brown-colored oil and toluene was evaporated to obtain the triethylammonium salt. The resulting product was then acidified with HCl to pH 2 and extracted with ethyl acetate. The organic layer was dried with magnesium sulfate, and the ethyl acetate was evaporated to obtain GMI.

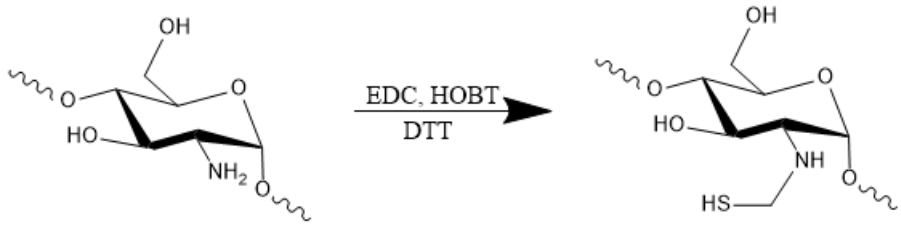
2.2.2. Synthesis of 4-arm PEG with maleimide groups (4-arm-PEG-Mal)



Scheme 2. Schematic of synthetic route for 4-arm-PEG-Mal.

Scheme 2 outlines the synthetic procedure for producing 4-arm-PEG-Mal. To summarize, a solution of 4-arm PEG 10K (1.00 g, 0.1 mmol) and GMI (0.074 g, 0.48 mmol) in dichloromethane was cooled to 0°C, after which a solution of DCC (0.107 g, 0.52 mmol) in dichloromethane was added. The resulting mixture was stirred at room temperature for 24 hours, filtered, and then the filtrate was precipitated using diethyl ether. The precipitate was subsequently dried under vacuum to a constant weight and stored at -20°C.

2.2.3. Synthesis of chitosan with thiol groups (chitosan-SH)



Scheme 3. Schematic of synthetic route for chitosan-SH.

Scheme 3 was employed to produce and characterize chitosan-SH, a chitosan derivative containing thiol groups. The synthesis involved dissolving chitosan (MW = 100 kDa) in DI water (5 mg/mL) and mixing it with three molar equivalents of cystamine dihydrochloride at pH 4.8. The carboxyl group of chitosan was activated using EDC and HOBT (at 3 equivalents) for two hours, and the conjugation process took place overnight. The resulting product was purified using dialysis tubing with a molecular weight cutoff of 10,000 kDa against DI water for three days. To break the disulfide linkage of the cystamine component, the solution was treated with TCEP at 5 equivalents and stirred for two hours. The pH was then adjusted to 3.5 with HCl, and chitosan-SH was precipitated in ethanol, re-dissolved in DI water, lyophilized, and stored at -20 ºC.

2.3. Hydrogel preparation

The 4-arm-PEG-Mal and chitosan-SH hydrogel precursors were dissolved in pH 7.4 PBS and then combined by mixing their respective solutions. Through a chemical process known as "clicking," the maleimide groups reacted with the thiol groups to create a three-dimensional structure, resulting in the formation of the gel.

2.4. Characterization

2.4.1. Characterization of GMI, maleimide and thiol hydrogel precursors

The molecular structures of GMI, maleimide and thiol hydrogel precursors were confirmed by Proton and Carbon Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR). ^{1}H and ^{13}C NMR spectra were recorded with an Avance Bruker equipped with BBO z-gradient probe. Experimental conditions were as follows: each sample scanned 128 times. The solvents used were (1) DSMO-d_6 for GMI and (2) D_2O for 4-arm-PEG-Mal and chitosan-SH.

2.4.2. Gelation time

The vial tilting method is utilized to determine the gelation time. In short, a solution of 4-arm-PEG-Mal and chitosan-SH hydrogel precursors in pH 7.4 PBS is prepared separately. Upon combining both solutions and quickly vibrating them at room temperature, the hydrogels are formed. The gel state is achieved if no flow is observed within a minute upon inverting the vial.

2.4.3. Swelling studies

A gravimetric method was employed to investigate the swelling behavior of freeze-dried hydrogels. The hydrogel samples were subjected to incubation at 37 °C in pH 7.4 PBS until they reached equilibrium weight. Excess water absorbed by the swollen hydrogels was removed using a filter paper, and the samples were weighed again. Next, the samples were dried until a constant weight was achieved. The swelling ratio was then calculated using the following equation:

$$\text{Percentage of swelling (\%)} = (W\text{wet} - W\text{dry})/W\text{dry} \times 100,$$

where Wwet and Wdry refer to the weight of wet and dry hydrogels respectively.

2.4.4. Degradation

To conduct degradation studies, we utilized weighted hydrogel freeze-dried samples. These samples were submerged in pH 7.4 PBS and kept at 37 °C for specific time intervals. After each interval, we removed the samples from the PBS solution, dried them, and recorded their weight. We then calculated the hydrogel degradation using the

following equation:
Degradation (%) = (*m*t/*m*0) × 100,

where *m*t and *m*0 refer to weight of freeze-dried hydrogels at selected time interval and before immersing in PBS respectively.

2.4.5. Rheological analysis

The Discovery Hybrid Rheometer is employed to analyze the dynamic rheological properties of hydrogels, utilizing parallel plate geometry with an 8 mm diameter. Frequency sweep measurements are conducted within the linear viscoelastic region, at a fixed strain and a temperature of 37 °C, ranging from 0.1 to 100 rad s^{-1}.

3. Results and discussion

3.1. NMR Analysis

3.1.1. GMI

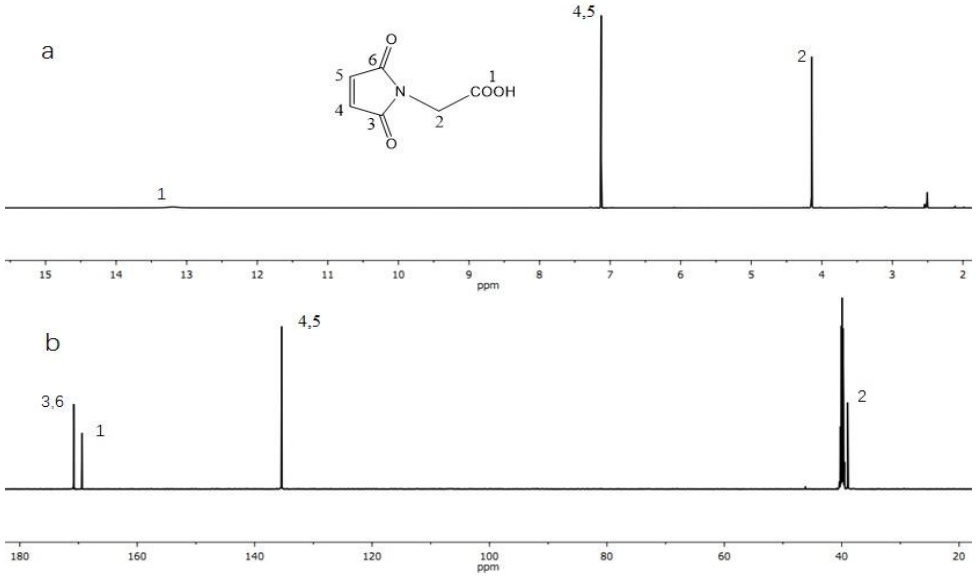


Figure 1. (a) ^{1}H and (b) ^{13}C NMR spectra of GMI in DMSO-d_6.

The NMR spectra confirmed the structure of GMI. Figure 1(a) shows the ^{1}H spectrum with peaks at approximately 13 ppm and 7.1 ppm, which are attributed to the -O-H bond of the carboxylic acid and the -CH=CH- bond of the maleimide ring.[2] In Figure 2(b), the ^{13}C spectrum displays peaks at 170.8 ppm and 135.3 ppm, corresponding to the carbonyl groups and double bond of the maleimide ring, respectively. Additionally, the carbonyl group peak of the carboxylic acid was detected at 169.3 ppm.

3.1.2. 4-arm-PEG-Mal

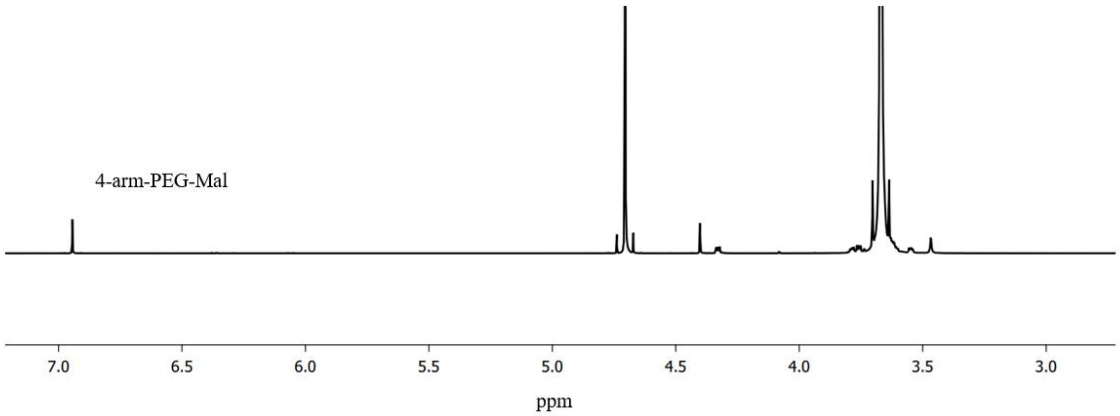


Figure 2. ^{1}H NMR spectrum of 4-arm-PEG-Mal.

Based on the 1H NMR spectrum shown in Figure 2, it has been confirmed that the 4-arm-PEG-Mal structure is present. Specifically, the peak observed at 6.9 ppm has been identified as protons in the maleimide, while the peaks at 3.8-3.4 ppm are attributed to protons in the 4-arm PEG backbone. By comparing the integrals of the resonance signals at 6.9 ppm and 3.8-3.4 ppm, it is estimated that the degree of functionality of the 4-arm-PEG-Mal is approximately 90%.

3.1.3. chitosan-SH

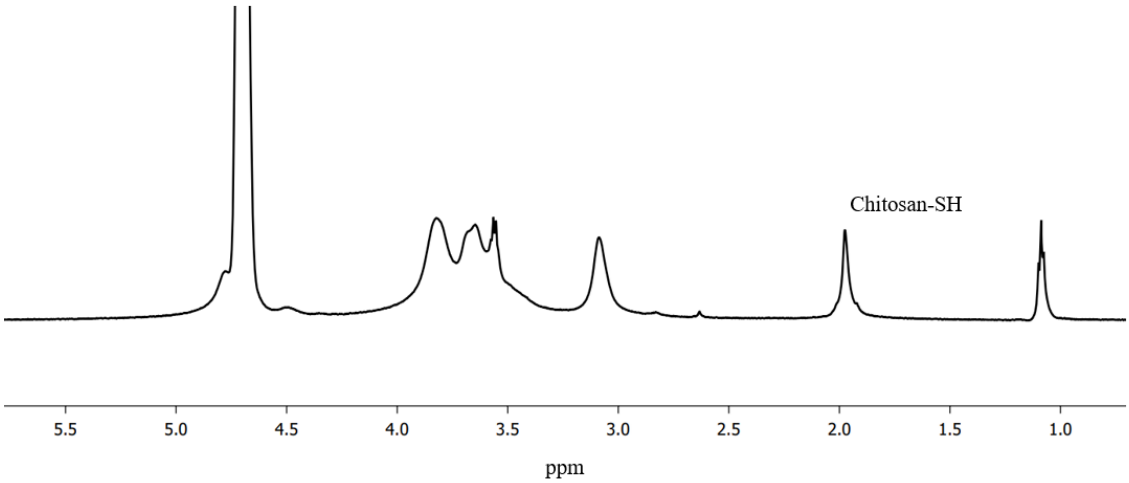


Figure 3. 1H NMR spectra of chitosan-SH.

The chitosan-SH structure was confirmed using 1H NMR analysis, which revealed the presence of cysteamine in the molecule through the observation of peaks at δ = 1.95 ppm. The modification of chitosan was determined by using the resonance of the acetamido group of chitosan as an internal standard at δ = 1.15 ppm. Additionally, the peaks observed at 4.0-3.0 ppm were identified as the protons in the chitosan backbone. By comparing the integrals of the resonance signals at 1.95 ppm and 4.0-3.0 ppm, it was estimated that the degree of functionality of the chitosan-SH was around 70%.

3.2. Gelation time analysis

chitosan-SH \ 4-arm-PEG-Mal	1%	2%	3%	4%
1%	No gelation	1h	30m	<1s
2%	1h	20m	10m	<1s
3%	30m	10m	≈ 10s	<1s
4%	<1s	1s	<1s	<1s

Table 1. Summary of hydrogels' gelation time.

Table 1 provides a summary of the gelation time of hydrogels, where the weight percent of hydrogel precursors ranges from 1% to 4%. The gelation time varies from no gelation (infinity) to ultrafast (<1 second). To ensure successful cell encapsulation, the only viable formula is the combination of 3% chitosan-SH and 3% 4-arm-PEG-Mal, as longer (>5 minutes) or shorter (<5 seconds) gelation times could lead to failure. Longer gelation times may result in a lower weight percent of hydrogel precursors used, leading to the formation of an amorphous gel. On the other hand, shorter gelation times could result in incomplete "clicking" of the maleimide and thiol groups, negatively impacting cell growth. Thus, the 3% chitosan-SH and 3% 4-arm-PEG-Mal formula was utilized for all remaining experiments and analysis.

3.3. Degradation analysis

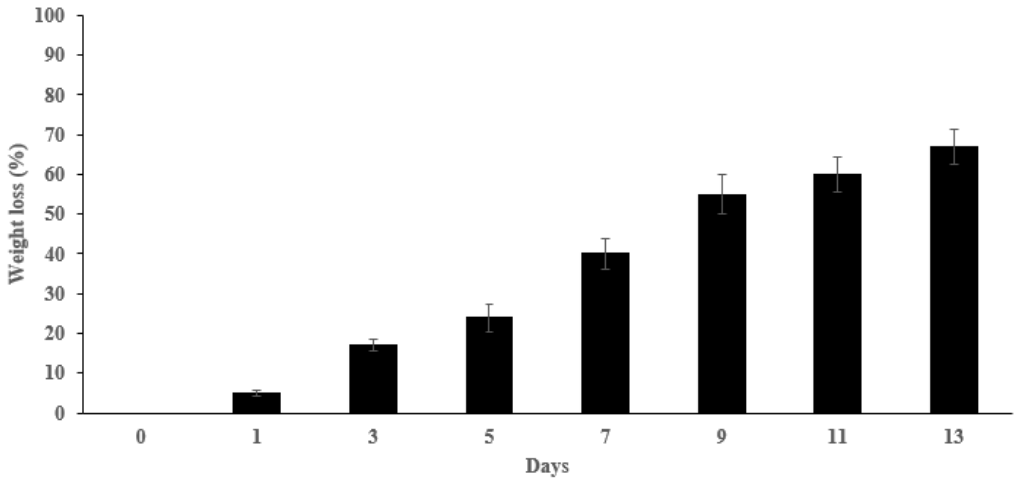


Figure 4. Weight loss of hydrogels at selected time intervals.

Figure 4 displays the rate of weight loss of the hydrogels. We measured the weight loss of hydrogels within 2 weeks. The hydrogels experienced a weight loss around 40% in the first week, after which they degraded steadily until the second week. Ultimately, the hydrogels degraded about 65%.

3.4. Swelling analysis

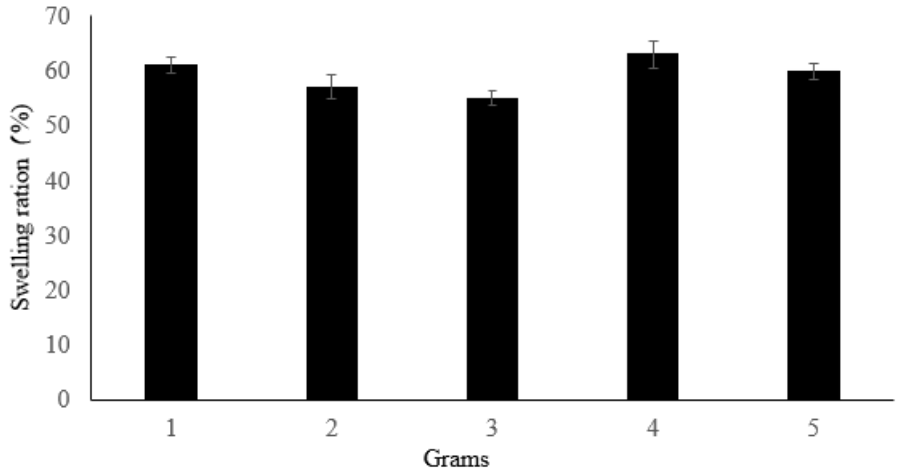


Figure 5. Summary of hydrogels' swelling ratio

Figure 5 displays the swelling analysis results for freeze-dried hydrogel samples, ranging in weight from 1 gram to 5 grams. Since each type of hydrogel exhibits distinct swelling properties, all 4-arm-PEG-Mal and chitosan-SH hydrogel samples exhibit similar swelling ratios, approximately 60%, despite varying weights. When submerged in PBS, hydrophilic samples absorb more water and produce a higher swelling ratio. Notably, 3% 4-arm-PEG-Mal and 3% chitosan-SH hydrogels absorbed 60% weight of freeze-dried hydrogel samples, indicating their high flexibility to mimic the extracellular matrix. Overall, these results demonstrate that the hydrogels generated using this formula exhibit exceptional flexibility.

3.5. Rheological analysis

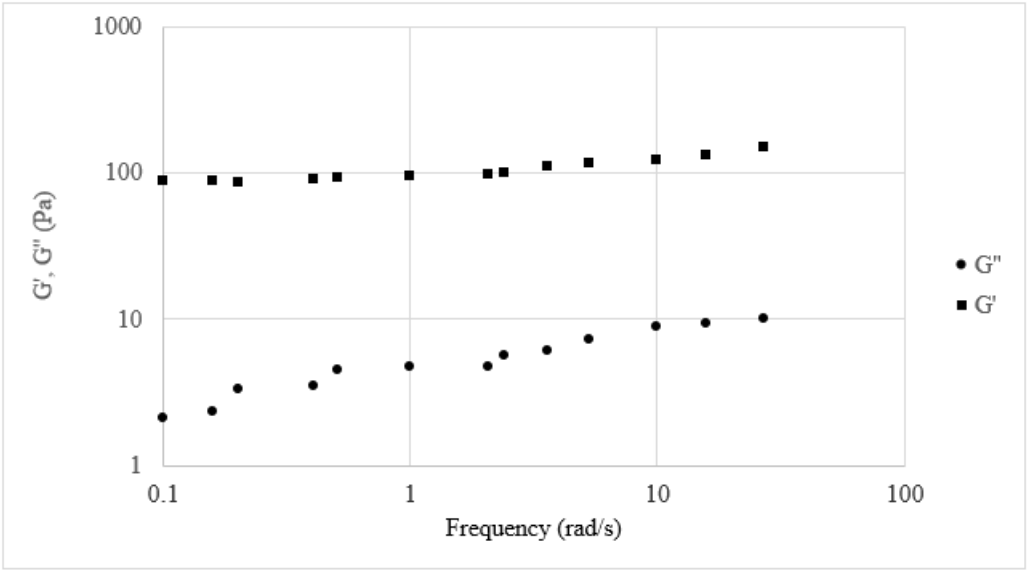


Figure 6. Frequency dependency of storage moduli (G′) and loss moduli (G″) of the hydrogels.

Figure 6 displays the relationship between angular frequency and the moduli G′ and G″ for hydrogels. The observed viscoelastic behaviors are characteristic of hydrogels. Specifically, the moduli G′ was consistently greater than G″, indicating that hydrogels formed via maleimide-thiol coupling chemistry exhibit elastic solid properties.

4. Conclusions

This article presents our research on the development of a new biocompatible hydrogel using precursors functionalized with 4-arm-PEG-Mal and chitosan-SH through the maleimide–thiol Conjugation. NMR analysis confirmed the successful synthesis of both precursors, and the hydrogels underwent several studies to examine their gelation time, swelling behavior, viscoelastic properties, and degradation rate. After conducting these studies, we found that the ideal formulas were 3% 4-arm-PEG-Mal mixed 3% chitosan-SH, which they also used for the remaining tests. Our results showed that the hydrogels were highly flexible, mimicking the extracellular matrix during the swelling study, and degraded about 65% after 2 weeks in the degradation study. Furthermore, the viscoelastic study revealed that the hydrogels produced through the maleimide–thiol Conjugation were elastic solids. Our research suggests that this new biocompatible hydrogel has great potential for biomedical applications.

5. References

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