Preparation of Injectable Composite Hydrogels by Blending Poloxamers with Calcium Carbonate-Crosslinked Sodium Alginate

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The effects of calcium carbonate-crosslinked sodium alginate on poloxamer hydrogels have been investigated. The mechanical strength, degradability, and thermal stability of hydrogels were characterized. The chemical and physical crosslinking in the composite hydrogels has resulted in an improvement of the compressive strength and elasticity of the hydrogels. These mixed hydrogels showed improved mechanical properties, elasticity, and stability as well as environmental responsiveness and injectability.

1. Introduction

Hydrogels have attracted extensive research interest due to their three-dimensional structure, high water retention, good mechanical properties, and excellent biocompatibility. Furthermore, intelligent injectable hydrogels can respond locally to environmental cues as well as adapt to the anatomical characteristics of the injection site, with applications in tissue engineering, sustained drug release, and other fields.[1,2] Currently, injectable hydrogels mainly composed of biopolymers, such as chitosan,[3] cellulose,[4] collagen[5] and polymeric materials such as polyethylene[6] and polyvinyl alcohol,[7] among others. Hydrogels are normally formed by physical or chemical crosslinking, or physical-chemical double crosslinking based on hydrogen bonding, ionic bond, and van't Hoff forces. Nevertheless, physically crosslinked hydrogels have low mechanical strengths and compression and shear resistances and are therefore unable to withstand the required loads. Therefore, improving the injectability and anatomic compliance of hydrogels along with achieving appropriate mechanical strength remain a major research focus. The majority of published research on this issue aims to achieve these properties by optimization of chemical synthesis methods to prepare the in situ hydrogels. However, chemical methods may lead to the presence of reactant residues, require complex preparation processes, be limited by material selection, and be difficult to produce industrially.

Poloxamers are triblock copolymers consisting of polyethylene oxide (PEO) and polypropylene oxide (PPO) with variable hydrophobicity or hydrophilicity properties based on the PEO and PPO block composition. As smart macromolecular materials, poloxamers are used to fabricate pH- and/or temperature-sensitive in situ hydrogels able to respond to different environmental conditions. Jung et al. fabricated thermosensitive injectable hydrogels based on poloxamers for sustained drug delivery.[8] Similarly, Samyr et al. described an organogel composed of oleic acid and poloxamer 407 (P407) together with sodium alginate (SA) to obtain an intravaginal drug delivery system and showed that incorporation with SA enabled the modulation of targeted drug release.[9] However, the low mechanical strength of pure poloxamer hydrogels limited their application under high compression and elastic conditions, as well as fast erosion in aqueous medium.[10]

To date, few studies have focused on improving the mechanical strength properties of poloxamers through combination with SA. SA is a polysaccharide extracted from natural brown algae that easily combines with divalent metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, etc.) to form a hydrogel with an 'egg'-box structure.[11] The most common iteration of these hydrogels is calcium ion-crosslinked SA hydrogels (Ca—Alg). And based on the chemical crosslinked method, SA hydrogels shows higher mechanical strength than other hydrogels and can be used for tissue repair,[12] drug-controlled release,[13] or cellular immune isolation.[14] The disadvantage of most Ca—Alg, however, is its lack of injectable properties due to its fast gelation time because of the Ca^{2+} source in Ca—Alg provided by a $CaCl_2$ solution, which leads to fast hydrogel formation. Theoretically, the use of a $CaCO_3$ solution, a weak electrolyte, would lead to a slower gelation rate given the slower release of Ca^{2+} ions. Nevertheless, few studies have focused on the formation of Ca—Alg hydrogels based on $CaCO_3$ as Ca^{2+} source. Based on former analysis, poloxamers mixed with SA and crosslinked with $CaCO_3$ was hypothesized might through chemical and physical double crosslinking to form an interpenetrating network (IPN) structure, with both a higher mechanical strength and injectable properties.

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To explore this hypothesis, we used P407, P188, and SA as the basic materials and $CaCO_3$ as the crosslinker to prepare a composite hydrogel by a mechanical stirring method. The mechanical strength, degradation of the composite hydrogels were assessed. Importantly, the mechanical strength of the composite hydrogels was analyzed through stretch and compression experiments. In addition, the crosslinking mechanism and interactions between the various molecules were assessed through rheological measurements. Finally, the cytotoxicity of the prepared composite hydrogels was assessed to evaluate their biocompatibility.

Experimental Section

Materials

SA (molecular weight 50–60 kDa) was purchased from the National Pharmaceutical Group Chemical Reagent Co., Ltd. P407 and P188 were purchased from BASF in Germany. Dimethyl sulfoxide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (Shanghai). $CaCO_3$ was obtained from Tianjin Kermel Chemical Reagent Co. Ltd. All other reagents were of analytical grade.

Preparation of Injectable Hydrogels

P407 and P188 were respectively dissolved in deionized water overnight at 4 °C to form homogeneous solutions. SA was completely dissolved in deionized water to prepare a 3 wt% solution. 10 mL P188 and 10 mL SA solution was mechanically stirred at room temperature, and coded as solution A. $CaCO_3$ was added into the P407 (10 mL) solution and stirred to form a homogeneous solution at a final concentration of 3 g/100 mL, and coded as solution B. Solutions A and B were then mix in a 1:1 ratio and stirred to form a composite hydrogel, followed by gelatinization at 37 °C for 2 h in a water bath. Based on previous experiments, P188 had little effect on the properties of the composite hydrogel, for which the current study focuses on the effect of P407 concentration of hydrogel formation and properties.

Morphology

The prepared composite hydrogels were frozen for 24 h and vacuum freeze-dried to constant weight at 60 °C. The samples were then gold coated and observed by scanning electron microscopy (SEM; S-4800, Hitachi) at an accelerating voltage of 20 KV to characterize the micro-morphology of the composite hydrogel sections.

Mechanical Properties

The mechanical properties of samples were measured using a Micro-electronic Universal Testing machine (LD1000, Shinco, China). The hydrogels were molded into rods of 2 mm in diameter and 5 mm in length for mechanical strength experiments. The samples were incubated in phosphate buffer solution (PBS; pH 7.4) for 24 h at room temperature prior to testing. All tests were performed at the tensile and compressive rate of 10 mm/min at room temperature. For the hardness experiments, the maximum force on the gel sample was measured by a plasmometer (SMSTA. XT Plus, UK). The thickness of the sample was 20 mm, and the test was performed using a P/0.5R type probe at 3.0 mm/s, with a compression deformation at 90% of the original height of the sample, at room temperature. The average value of five replicates was taken.

Rheological Assessment

The relationship between hydrogel macroscopic behaviors and materials microstructure and other physicochemical properties can be described by rheology experiment. In the linear viscoelastic range, rheological experiments were conducted using a CVO Rheometer (ARES G2, TA instruments, USA). The frequency sweep was performed within the range 0.1 rad/s to 500 rad/s at 25 °C and 1% strain. All experiments were performed using a 45 mm plate and a 5 mm gap.

Stability of Composite Hydrogels

The *in vitro* stability of the composite hydrogels was assessed by weight loss method. Samples with initial weight W_0 were immersed in PBS solution (pH 7.4) and shaken at 37 °C at an oscillation frequency of 10 rmp/min. After 0, 1, 3, 10, 20, 30, and 40 days, the hydrogels were weighed (Wt) after the surface liquid had been lightly swabbed off. To compare the difference stability of the composite hydrogels in different solutions, same experiments were also conducted in fetal bovine serum (FBS) solution.[15,16] All the measurements were performed in triplicate.

Thermogravimetric Analysis

The thermal properties of *in situ* hydrogels were assessed by thermogravimetric analysis (TGA; L75, Germany). The raw materials and freeze-dried sample powders were accurately weighed at 4.0 mg and scanned at 10 °C/min under a N_2 atmosphere from 25 °C to 700 °C.

MTT assay of Hydrogels

The biocompatibility of the composite hydrogels was assessed by the MTT assay using fibroblasts (L-929). The test was conducted according to the literature of Rejinolda.[17] Briefly, the absorbance value was detected at 490 nm and the cell viability (CV) was calculated by Eq. 1:

$$CV = \frac{ODtreated - ODblank}{ODcontrol - ODblank} \times 100\%$$ (1)

where OD_{blank} is the absorbance of the blank medium, $OD_{control}$ is the absorbance of the medium, and $OD_{treated}$ is the absorbance of samples.

2. Results and Discussion

According to our previous experimental results, the higher the concentration of SA solution, the faster the response time and the stronger the strength of the Ca—Alg hydrogel. However, at SA concentrations above 3.5 g/mL, the solution was too viscous for injection. Therefore, a 3 g/mL SA solution was used in all subsequent experiments. The concentration of P188 had little effect on the responsiveness of the composite hydrogel, therefore, a P188 concentration of 5 g/mL was selected according to

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previous experiments. In addition, the concentration of P407 had obviously effect on the responsive time and formation of the composite hydrogel. The phase transition time is only 10 s when the concentration of P407 was above 28 g/mL, resulting lacking the injection time of the composite hydrogel and the result was consistent with the experiments of Hu et al.[18] On the contrary, at P407 concentrations lower than 10 g/mL, the composite hydrogel lacked temperature responsiveness to the environments. Based on our previous experiments, the gelation time of composite hydrogel was about 150 s–320 s at the 37 °C with P407 concentration of 10–15 g/mL, indicating that the composite hydrogel had not only the responsiveness ability but also injectable property. Therefore, only the P407 concentration was varied in the composite hydrogel, at 10, 15, 20, and 25 g/mL in experiments, named H1, H2, H3, and H4, respectively.

2.1. SEM haracterization

The four composite hydrogels were assessed by SEM (Figure 1). All four hydrogels had obvious pore morphology albeit with a different microstructure and different from each other. And the homogeneous state indicating good compatibility of those raw materials. The H1 sample (Figure 1a) had smooth inner surface and a three-dimensional porous structure with a uniform pore diameter and various interconnecting pores. The cross-section SEM images of composite hydrogels of H2, H3, and H4 (Figure 1b–d) showed a variation in the pore diameter. These hydrogels had a larger average pore diameter and a more heterogeneous structure than H1, indicating that the P407 concentration played an important role in the cross-linking process and affected the hydrogel microstructure. From Figure 2c, and 2d, a few areas of H3 and H4 hydrogels exhibited dense structures and string-like structure, likely due to the

presence of excess P407 molecules formed columnar as exhibited in Figure 1, which would hinder the diffusion even of small molecules such as water within the hydrogel. Thus, a higher concentration of P407 may lead to steric hindrance on the formation of a micro-network structure, leading to the formation of multiple channels in H3 and H4 hydrogels.

The results indicated that the interaction of materials' molecules might contributed to the interpenetrating network (IPN) structure formed in the composite hydrogel. And the IPN structure composed of chemical crosslinking of the Ca–Alg network and a physical crosslinking interaction between P407 and P188 was assumed to strengthen the entanglement between different molecules (Figure 2b). Additionally, as Ca^{2+} is released slowly in the $CaCO_3$ solution, the Ca–Alg hydrogel network forming rate corresponded to the formation of a P407 and P188 network structure, resulting in more crosslinks spots built in H1 than for the remaining three hydrogels. The microporous structure of H1 had the characteristics required for the diffusion of drug or bioactive molecules, able to act as a controlled release drug material or as a potential carrier of the different materials Poloxamers have a unique hydrophilic and hydrophobic core-shell structure, with the gelatinous properties being highly dependent on their concentration and temperature. Following an increase in temperature and/or concentration, the hydrophobic groups of PO wrap within the internal structure, while the hydrophilic EO groups orientate externally and interact with water molecules, leading to a micellar structure poloxamer hydrogel (Figure 2a). Especially, poloxamer hydrogels are easily formed at a concentration of 15.0–30.0%.[19] Lacking stability of pure poloxamer hydrogel, however, poloxamer was common combined with other biomaterials to fabricated composite hydrogel. Herein, SA was mixed with poloxamers and cross-linked with Ca^{2+} to form a dual physicochemically crosslinked structure (Figure 2b).
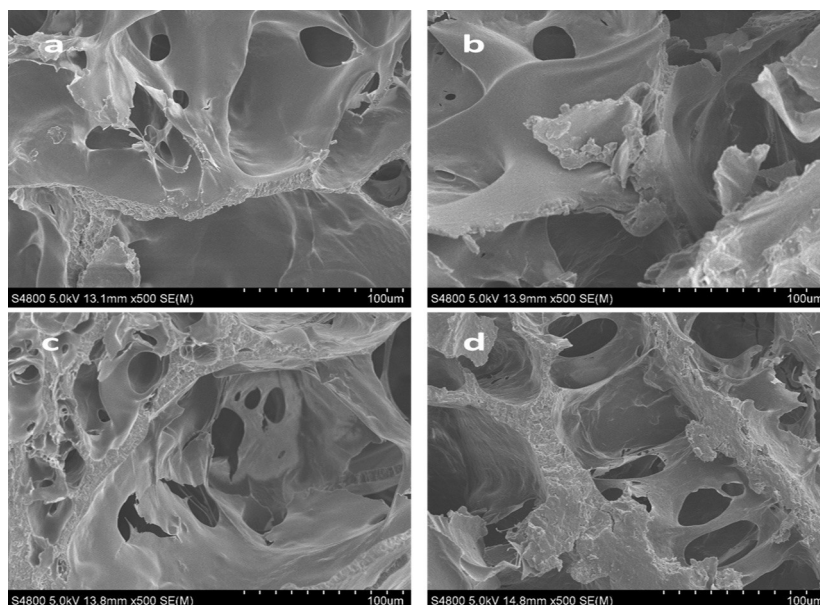

Figure 1. Cross-sections SEM images of composite hydrogels. (a H1, (b) H2, (c) H3, (d) H4. Scale bar: 100 μm.

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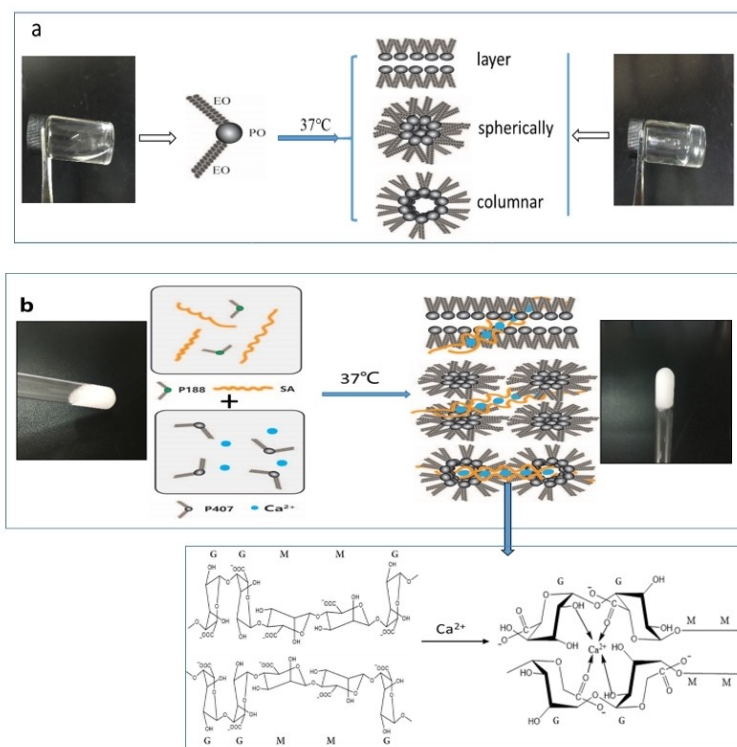European Chemical
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Figure 2. Schematic diagram of the reaction. (a) Poloxamer gel formation with different microstructure (layer, spherically, columnar structure) upon increasing temperature. (b) Schematic diagram of the composite hydrogels.

2.2. Mechanical Properties

The compression modulus as well as their stress–strain curves were assessed and the results were showed in Figure 3. From the Figure 3a, stress was much higher for H1 and H2 than for H3 and H4, at 1.305 and 1.175, 0.802 and 0.525 KPa, respectively, with a corresponding elongation at break of 263.9%, 230.3%, 142.2%, and 49.4%. Thus, four hydrogels revealed different elongation break because of different concentration of P407. Theoretically, the higher the P407 concentration, the greater the number of crosslinking degree. However, the results showed that a high P407 concentration might leads to an increase in the space resistance between molecular chains, hindering inter/intra molecular interactions, finally affecting the macroscopic properties such as mechanical strength, and the results was corresponding with the SEM analysis. Compared to the remaining hydrogels, the tensile strength of H4 was weak since the high concentration of P407 obviously affected the formation of intermolecular interactions.

Compression modulus is refers to elastic properties of materials and reflects the material's ability to resist elastic deformation. From the compressive modulus perspective, the higher the elasticity modulus, the greater resistance and stiffness of material to external forces; conversely, the lower the modulus of elasticity, the more easily the material deforms and in the result the materials is difficult to maintain the original shape. The compression modulus of the four samples was tested by universal electronic tension machine after gelation for
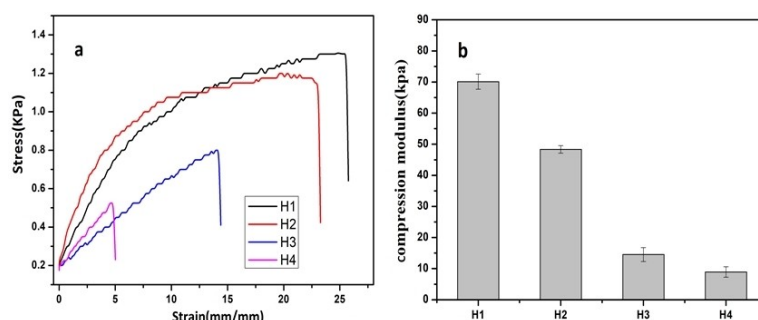

Figure 3. The mechanical strength (a) and compressive modulus (b) of samples.

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12 h (Figure 3b). The compression modulus of H1, H2, H3, and H4 samples was 74.52, 46.25, 14.53, and 8.92 kPa, respectively, showing that H1 had a greater resistance and stiffness as well as the ability to retain its structure following external forces.

Different from the compression modulus, the hardness of the hydrogel reflects the resistance of the composite hydrogel to external forces, as well as the softness of the hydrogel. Generally, the higher of mechanical strength, the harder of hydrogel, with stronger ability to resistant external forces and less softness.[20] For application in the human body, the appropriate mechanical strength as well as the softness of composite hydrogel are both important. Furthermore, composite hydrogel prepared by biomaterials might degrade in the body with the time resulted the hardness decreased. Based on this, the hardness change of four hydrogels with time were conducted and the result showed in Figure 4a. From the Figure 4a, the firmness of the four samples increased with gelation time, reaching the highest value at about 96 h. The hardness of the H1 composite hydrogel was obviously higher than that of the remaining samples, at ~1.5 times that of H2 and H4. The hardness of all samples decreased after ~120 h, except for H1. Form another compressive testing, H1 showed an obvious compressibility after the object removed (Figure 4: c1, c2). On the contrary, the Ca–Alg hydrogel had a high hardness to deformation and a weak elastic property (Figure 4: d1, d2), while pure poloxamer hydrogels are based on physical crosslinking, resulting in weak mechanical strengths to resist the external force (Figure 4: b1, b2).

In all, the results indicated that H1 has a higher mechanical strength, stability and better flexibility, likely due to not only the combination with SA but appropriate concentration of P407 to form multifunctional forces between molecular chains in composite hydrogels (Figure 2).Based on the interpenetrating crosslinked system, many crosslinked or entanglement spots were formed in the IPN structure resulting the interaction between the polymer molecule chains to enhance the compressive strength and elastic. For the composite hydrogel, the mechanical difference between hydrogels was mainly due to the effect of P407 molecules on the microstructure with its varying concentration. Theoretically, the higher the concentration, the stronger the gelatinization in the composite hydrogel. However, as discussed above, the composite hydrogel showed higher mechanical strength and elastic properties at lower P407

concentrations. According to previous results, the properties of poloxamer molecules and their intermolecular interactions play an important role in the formation of structures and affected the properties of the composite hydrogel. Although, SA crosslinked with Ca^{2+} occurs through ionic gelation based on chemical reaction and results in a high mechanical strength, albeit with an accelerated coagulation rate which limited injection property. Combined with poloxamers and $CaCO_3$ as crosslinking agent, the mechanical strength of the composite hydrogel was thus improved and also obtained a certain flexibility.

2.3. Rheological Characteristics

Polymer rheology is the research of polymer liquid mainly refers to the nonlinear viscoelastic behavior of polymer molecule solution in flow state. In addition, the relationship between this behavior and material microstructure and other physicochemical properties. Furthermore, the behavior of materials at microscopic level determines the intrinsic physical mechanism of macroscopic behavior. The storage modulus (G′) is also called elasticity modulus and represents the energy stored in viscoelastic materials based on elastic deformation. While loss modulus (G″) is also called viscous modulus, it refers to the material deformation due to viscous deformation (irreversible) and loss of energy, reflecting the material viscosity.[21] Normally, the value and the relationship of G′ and G″ in experiments reflected the rheology property of materials.

Rheological studies of four composite hydrogel were performed in the linear viscoelastic range from 0.01 to 100 Pa at a frequency of 1.0 rad/s. Based on previous results, all experiments were conducted at a strain of 1%. The rheological properties of the four gels, including the energy storage modulus (G′) and loss modulus (G′) of the composite gels, were studied by dynamic oscillation scanning mode (Figure 5). The ratio of G′/G″(tanδ) represents the loss factor, where the larger the value, the greater the viscosity ratio of the system. From Figure 5, the storage modulus G′ and the loss modulus G″ of all samples showed a frequency dependence wherein, with increasing frequency, both G′ and G″ increased (Figure 5a). Furthermore, G′ values of the four samples were higher than the G″ values within the scope of the oscillation frequency of 0–
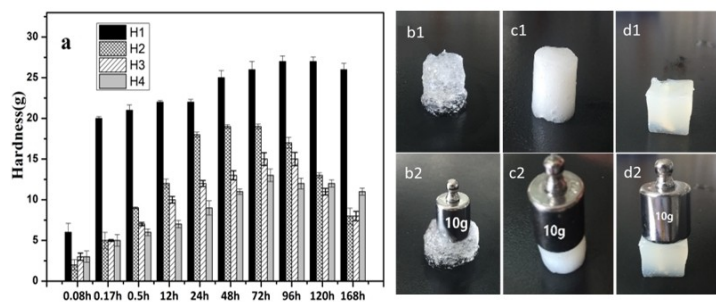

Figure 4. The change in hardness with time (a) and the compressive results of pure poloxamer hydrogel (b),H1 (c) and Ca–Alg hydrogel (d). (1: before compression, 2: after compression).
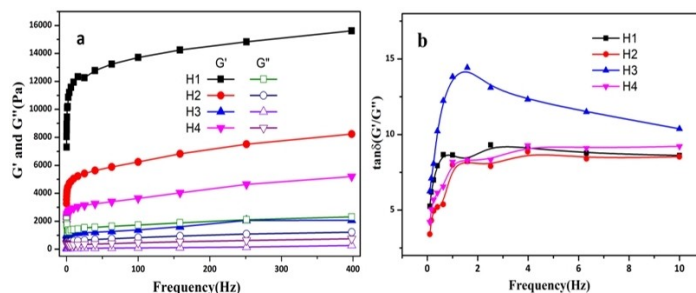
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Figure 5. The relationship between the frequency and the storage modulus G′, loss modulus G″ and tanδ of the four hydrogels.

10 Hz, indicating an elastic behavior. In addition, the G′ value of H1 was much higher than that of the remaining hydrogels, likely due to the chemical crosslinking of the Ca—Alg gel forming an interpenetrating structure with the poloxamer hydrogel network as well as leading to entanglement with poloxamers and SA macromolecules at the concentration 10 g/mL of P407. As a result, the intensity of crosslinking of the whole network structure of H1 increased, but the mobility between the molecular chains decreased. However, the higher the concentration of P407, the lower the mechanical strength of the hydrogel, possibly due to the fact that un-crosslinked P407 molecules prevent cross-linking inter/intra interaction of macromolecular chains.

The G′/G″ ratio of the four gels at an oscillation frequency of 1–10 Hz showed that the tanδ value of H4 increased obviously with increasing frequency, followed by a decrease at ∼1.8 rad/s (Figure 5b); conversely, the remaining gels exhibited an increase with increasing frequency and then remained unchanged. The result indicated that as the concentration of P407 increased, the viscosity of the mixed hydrogel increased accordingly. Viscosity is a measure of the adhesiveness of a fluid. High viscosity indicates greater internal friction, molecules motion resistance and the loss of system energy. Intramolecular and/or intermolecular interactions might play an important role in the linear viscoelastic behavior of polymers.[22] And the excess P407 molecules in the higher concentration may have contributed to the higher viscosity and weak crosslinked function formed, as stated above.

2.4. Degradation Experiment of Composite Hydrogels

Due to the effects of retention time in vivo and the presence of degradation products, the biodegradation rate and degree are important for most medical biological materials. In other words, the degradation process reflects the stability of the hydrogel. The degradation profiles of the four hydrogels were assessed in PBS and FBS at 37 °C (Figure 6) for the application in the body. From the Figure 6, the weight of hydrogels decreased significantly with time in the PBS solution (Figure 6a). H2 and H3/H4 were almost break down into blocks after 15 h, respectively. The degradation rate of H1 on day 1 was 23.76 %. The degradation process of four hydrogel in FBS showed a similar trend, albeit with a longer complete decomposition time than that of PBS solution. However, the H1 degradation ratio of H1 was 34.37 % on day 3 in FBS, which means that the degradation time of H1 was longer that of others in both conditions, indicating a better stability of H1. Unlike other hydrogels, the weight of H4 in both solutions increased slightly in the initial phase, then decreased with time increased.

The degradation of the hydrogel is mainly due to the fact that the solvent enters into the three-dimensional structure, breaking the crosslinking point and weakening the interaction of macromolecules. Furthermore, the stability of the four hydrogel samples differed between FBS and PBS likely due to the variation in solutes type and concentration. Solutes such as mucin, albumin, and sugar changed the viscosity of the FBS solution and affected the degradation process. For H4, the phenomenon of weight increased at first and then decreased in solution might be due to its high content of P407, leading to
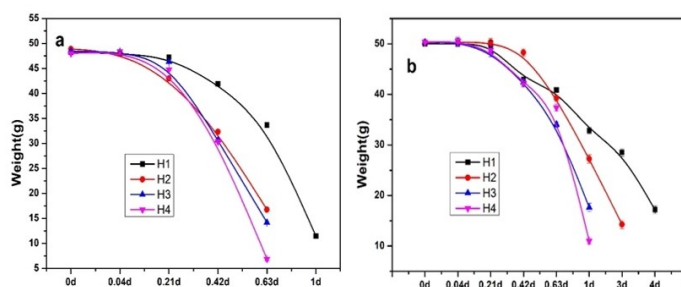

Figure 6. The degradation of composite hydrogels in (a) PBS and (b) FBS solutions.

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stronger water absorbing capacity and eroded phenomena which corresponding with the results of Rehman's.[11]

2.5. Thermogravimetric Analysis

According to the preliminary analysis results, subsequent experiments were only performed using H1 because of higher mechanical strength and stability. The TGA curves of P407, P188, SA and sample showed that that the decomposition of the hydrogel occurred in two steps (Figure 7a), the first step of P407, P188 and H1(sample) were occurred between 300 °C and 450 °C, with a decomposition temperature of 382.16 °C. The weight loss rates were 98.12%, 97.01% and 65.44%, respectively. The step was assigned to the loss of binding water of the materials and composite hydrogel and the fracturing of the glycoside bond. For SA, the decomposition temperature is between 200 °C and 300 °C,[23] indicating that when blended with poloxamer, the decomposition temperature of H1 is significantly higher than SA. The second step occurred between 600 °C and 700 °C, with a decomposition temperature of 664.06 °C and a weight loss rate of 8.39%. Therefore, compared with the raw materials P407, P188 and SA, the stability of the composite hydrogel has been significantly improved. It is shown that the cross-linked state and a certain cross-link density in the mixed gel can improve the thermal stability of the composite hydrogel. In addition, consistent with the previous analysis results, the microstructure composed of appropriate P407 concentration may play an important role in the formation of the interpenetrating network and affect the stability of the composite hydrogel.

2.6. MTT Assays

The cytotoxic effects or biocompatibility of a composite hydrogel following injection is amongst the most important properties of a biomaterial. The MTT assay was used to evaluate the composite hydrogel biocompatibility. Analysis of MTT test results showed that the three raw materials were non-toxic, with cell proliferation rates of 97%, 98%, and 97%, respectively (Figure 7b). The cytotoxic effects of H1 upon decomposition at 0, 1, 10, 30, and 40 days were assessed (H1-0, H1-1, H1-10, H1-

30 and H1-40, respectively). The cell proliferation rates were 95%, 93.2%, 92.1%, 93.2%, 91.1%, and 90.7%, respectively (Figure 7b), which were slightly lower than for the raw materials though above 80%, indicating that the composite hydrogels prepared through this experiment had non-toxic effects and good biocompatibility.

3. Conclusions

Herein, composite hydrogels were prepared based on poloxamers combined with SA. The obtained hydrogels possess a higher mechanical strength and flexibility as well as injectability property through the formation of a multiple force crosslinked structure between Ca—Alg and the poloxamer hydrogel. $CaCO_3$ was adopted as the Ca^{2+} source in order to slow the gelation rate of Ca—Alg. The best injectable in situ hydrogel was obtained using 15 g/ml of P407 and 2 g/ml of P188, and $CaCO_3$ as the crosslinking agent, through the blending of 3 g/ml of SA. As a weak electrolyte, $CaCO_3$ allows the slow release of Ca^{2+} in solution, contributing to the control of the SA hydrogel formation rate. With physical crosslinking by intramolecular and intermolecular hydrogen bonding as well as van der Waals forces between the molecules, poloxamer and Ca—SA gels constitute the association structure of multiple sites. According to the analysis, the resistance to external pressure of the composite hydrogels was enhanced, with a compression modulus of 75.32 kPa, considerably increased compared to that of pure poloxamer hydrogels. Furthermore, the flexibility and stability were also improved compared with those of Ca—Alg hydrogels. Finally, the composite hydrogels showed no cytotoxic in the MTT assay.

Thus, the prepared composite hydrogel showed not only improved mechanical strength, elasticity, temperature responsiveness, and injectability, but also a simple preparation process, material availability, environment friendliness, making it a potential in situ hydrogel for clinical use. However, the shear performance needs to be improved for its application as an injectable gel in specific human organs or tissues, especially knee joints. Therefore, further research should focus on methods to improve the shear resistance while improving the compressibility of these hydrogels.
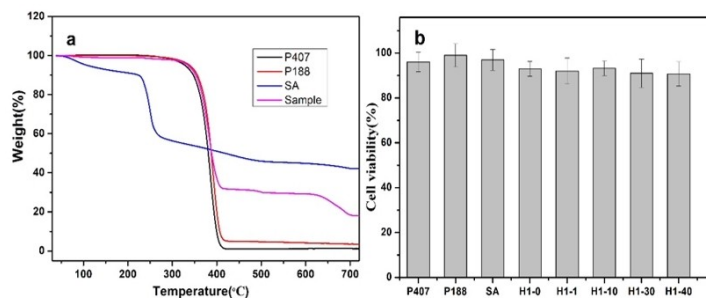

Figure 7. Results of TGA (a) and MTT (b) of H1 and its degradation products at different times (H1-0: 0 day; H1-1: 1 day; H1-10: 10 days; H1-30: 30 days; H1-40: 40 days).

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Conflict of Interest

The authors declare no conflict of interest.

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