Injectable Glucan/Alginate/poly(γ-glutamic acid) Gel for Bone Formation and Differentiation

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ABSTRACT

Aqueous mixture of β-glucan, alginate and calcium form poly(γ-glutamic acid) (Ca-γ-PGA) was compared with the traditional hydrogels cross-linking with calcium chloride and sodium form γ-PGA. The suitable proportion by investigating the characters of swellability, pH sensitivity, retentiveness, drug release mechanism and the hemocompatibility of each hydrogels, compared with alginate hydrogel only. The addition of glucan made the hydrogel film more ductile than alginate with Ca-γ-PGA one. The swelling ratio and the wetting ratio also increased with the glucan one. The results indicated that calcium alginate hydrogels may be useful for the localized delivery of drug in the intestinal environment. In addition, all the data shows that, the properties of the material with Ca-γ-PGA hydrogel were better than calcium chloride and sodium form γ-PGA. Both CaCl₂ and Ca-PGA mixed alginate/glucan exhibited almost no adsorption of human serum albumin (HSA), whereas the adsorption of human plasma fibrinogen (HPF) of CaCl₂ way was 10 times of that of Ca-PGA one. Cell counting and the MTT analysis showed a significant increase in the number of MG-63 osteoblasts with Al-PGA gel. Therefore, a gel form of alginate, glucan and Ca-γ-PGA is a biocompatible material and could open up a clinical application to mankind.

Keywords: β-glucan, alginate, calcium form poly(γ-glutamic acid) (Ca-γ-PGA), Wound dressing, controlled-release

1 INTRODUCTION

Alginates is used extensively as a hydrogel component these days, because of its many advantages such as hydrophilicity, high swellability, non-toxicity and easy-preparation. Alginate (Alg) consists of linear polymer of two monomers, β-1, 4-D-mannuronic acid (M) and α-1, 4-L-glucuronic acid (G) residues joined by β-1,4 and α-1,4 glycosidic bonds. Poly(1-glucuronic acid) sequences dimerize in the presence of divalent cations (e.g., Ca²⁺, Zn²⁺, Cu²⁺) to form hydrogel network. It is poorly water-soluble in low pH conditions, such as in the stomach, and dissolvable in alkaline environments, such as in the intestines, as a result of its acidic groups exhibit the alteration of COOH/COO⁻ thus the pH-sensitive crosslinked alginate hydrogels are currently receiving deals of interests for utilizing in drug delivery systems.

Polyglutamic acid (γ-poly glutamic acid, γ-PGA) is a natural polymer polymerized linearly copolymerized from the amino acid glutamic acid which formed peptide bonds between the amino group of GA and the carboxyl group at the end of the GA side chain.

(1-3),(1-6)-β-Glucan is a water-soluble and biodegradable polymer fermented from plant incubation. β-Glucan can boost the immune response by activating macrophage cells. It consists of β-(1-3) linked-d-glucose residues with oneβ-(1-6) linked d-glucoyl group for every three glucose residues. β-Glucan is effective against allogeneic, syngeneic and autochthonous tumors. It shows antibacterial and antiviral effects and exhibits the wound healing activity (Lee et al., 2003; Bohn and BeMiller, 1995).

In the last few years the attention has been focused on the study of bioartificial materials based on polysaccharides. Polysaccharides are biological polymers that can be obtained from different sources: microbial sources such as dextran and gellan, animal sources such as chitosan, and vegetal sources such as starch. Several advantages can derive from the use of these macromolecules. However, studies on glucan in biomedical applications are scarce. The only one was using sponges of glucan and gelatin for artificial skin (Lee et al., 2003).
The aim of this work is to study the wound dressing applicability of glucan-blending Alginate-Ca-PGA hydrogel films prepared via casting method. There are two advantages using this blend as wound dressing. Glucan would be released from the blend film during the healing process. Glucan is reported to be promoting cell proliferation (Lee et al., 2003). Thus releasing glucan would be able to accelerate the healing of wound. Furthermore, the Alg-Ca-PGA hydrogel can provide the mechanical strength of wound dressing, thus the Alg-Ca-PGA/glucan film would be more suitable for wound healing applications than in hydrogel form. In this work, these Alg-Ca-PGA/glucan films were subject to hydrophilic tests, tensile tests, protein adsorption, and blood coagulation tests. The results of this work would demonstrate the applicability of Alg-Ca-PGA/glucan film as a wound dressing.

2 MATERIALS AND METHODS

Materials

β -Glucan stock (11wt.%) was purchased from Geneferm Bio Technology Co. Ltd. (Taiwan) which was from mushroom strain fermentation with a wide distribution molecular weight of about 2MDa. Sodium alginate (about 22000) was purchased from Arcos Organics, USA. Ascorbic acid 2-glucoside (AA2G) was purchased from Vedan Enterprise Co., Taiwan. Calcium chloride was purchased from Shimakuy Chemical Co., Japan. Human serum albumin (HAS) (Mw: 66000) and human plasma fibrinogen (HPF) (Mw: 341000) was purchased from Calbiochem, USA.

2.1 Preparation of gel samples

A 1.5wt% alginate solution was prepared by dissolving sodium alginate powder in deionized (DI) water at room temperature for 24 h under stirring to form homogeneous solution. Glucan powder was dissolved in 5wt% acetic acid at room temperature under stirring for 24 h to form a homogeneous solution of 1wt%. Calcium PGA powder was dissolved in DI water at room temperature under stirring for 2 h to form a homogeneous solution of 3wt%.

Afterwards, 20ml of Alg solution was cast onto a glass plate and air-dried at 25 °C for 1 d. Then 20ml of the glucan solution was poured over the dry sodium alginate film and air-dried at 25 °C for 1d. Finally, 20ml of the Ca-PGA solution was poured over the dry film to form a hydrogel film. The resulting hydrogel was labeled as Alg-Glu-Ca-PGA. For comparison, dry sodium alginate film was soaked in either 10wt% CaCl2, 3wt% Ca-PGA or 3wt% Na-PGA aqueous solution. The resulting hydrogels were labeled as Alg-CaCl2, Alg-Ca-PGA and Alg-Na-PGA, respectively. All these hydrogel films were rinsed with DI water to remove residual solutions. Then these hydrogel films were dried at 60 °C for 1d in an oven.

2.2 swelling ratios test

To measure the swelling behavior of the films, samples of 1cm×1cm were dried in an oven for 2 h at 105 °C. Afterwards, the samples were placed in a humidifying chamber of 90% relative humidity at 37 °C, and weighed at specific time points. The swelling ratios of the samples were calculated as follows:

\[ SR = \frac{W_{wet}}{W_{dry}} \]  

Where \( W_{wet} \) and \( W_{dry} \) represent the weights of the film in the wet and dry states, respectively.

2.3 Water retention capacity test

A piece of swollen hydrogel was weighted (\( W_i \)) and placed in a tube. After centrifuging the tube at 2000, 8000, or 16000 g at 25°C for 5 min, the sample was carefully removed and weighted (\( W_t \)). The water retention (WR) was calculated using the following formula:

\[ WR\% = \frac{W_i - W_d}{W_i - W_t} \times 100 \]  

where \( W_d \) is the dry weight of the sample.

2.4 Compression modulus measurement

Effective crosslinking density of hydrogels was calculated from the compression modulus at the equilibrium swelling state in distilled water. The specimens were cut into disks of 25 mm in diameter. The equilibrium heights of swollen gel were recorded and the compression modulus was measured using a universal testing machine (Chun Yen Testing Machines Co. Ltd., Taiwan). The deformation rate was 5 mm/min and the extent of the deformation was set at 1/5 of the initial height of a swollen hydrogel. The compression modulus was determined from

\[ F/A = -G(\lambda - \lambda^2) \]  

where \( F/A \) is the compressive stress applied, \( G \) is the compression modulus, and \( \lambda = 1/10 \), where \( I \) and \( 0 \) are the heights of deformed and original gels, respectively. The crosslinking density was calculated based on the theory of rubber elasticity

\[ n_c = G/RTv_{2s}^{1/3}a_0^2 \]  

where \( n_c \) is the effective crosslinking density in mol/m^3, \( R \) is the gas constant, \( T \) is the absolute temperature, \( v_{2s} \) is the polymer volume fraction of a hydrogel at equilibrium swelling, and \( a_0 \) is the isotropic dilation factor and is approximated to \( v_{2s}^{1/3} \) (where \( v_{2s} \) is the polymer volume fraction at the relaxed state).

2.5 Drug release test
A piece of hydrogel film of 3×3 cm² were immersed in 50 ml aqueous solution of 200 ppm AA2G at room temperature for 12 h in a shaking bath (100 rpm). The membranes were gently taken out of shaking bath and dried at 37 °C for 12 h. In order to investigate the mechanism of empirical release from the crosslinked alginate membranes, the Ritget-Peppas assumes a time-dependent power law function.

\[
\frac{M_t}{M_\infty} = k t^n
\]  

where \(k\) is a kinetic rate constant, \(t\) is the release time, and \(n\) is a diffusional exponent. This power law is only valid for the first 60% of the release profile. For a slab-based delivery system, when \(n \leq 0.5\), the release mechanism is Fickian diffusion. When \(n = 1\), relaxation controlled occurs, leading to zero-order release. When the value of \(n\) is between 0.5 and 1, the release follows non-Fickian diffusion, where the system will be diffusion and relaxation controlled.

### 2.6 Adsorption of proteins

The adsorption of HAS and HPF was measured. A piece of hydrogel film of 1×1 cm² was immersed in 5 ml of pH 7.4 PBS containing 2 mg/dl HSA or HPF at 37°C for 24 h under 100 rpm shaking. Afterwards, the samples were gently taken out and rinsed five times with PBS, followed by placing in 1 wt% aqueous solution of sodium dodecyl sulfate (SDS) and shaken for 60 min at room temperature to remove the protein adsorbed on the surface. The protein content of each sample was measured using the BCA reagents (Pierce). The absorbance at 562 nm was measured using a spectrometer to calculate the concentration of protein.

### 2.7 Evaluation of platelet adhesion

The determination of platelet adhesion and thrombus formation was following the procedures given in the literature. Briefly, platelet rich plasma (PRP) (100 µL) was placed on test hydrogels (1×1 cm²) at 37°C for 30 and 60 min, respectively.

The baseline platelet count (about 4×10⁷ µL⁻¹) and that after adhesion were measured using a hematology analyzer (CA-620, Medonic, Sweden). The extent of platelet adhesion relative to the PRP control was calculated as follows: Platelet adhesion (%) = \(\frac{n_0 - n_t}{n_0} \times 100\) (6)

### 3 RESULT

**Crosslinking density comparison between three kinds of alginate hydrogel**

As known, Ca²⁺ acts as crossing bridge that triggers the crosslinking and formation of hydrogels, but the higher Ca²⁺ concentration, the worse the hydrogels. Alginate has well hydrophilicity and high swellability originally, but it is only in the aid of Ca²⁺ that alginate formed film. Since that, Ca²⁺ might eliminate the well properties of alginate during gelation process. To figure out the problem, we investigated spectacular hydrogel formed by mixing γ-PGA with alginate, Ca²⁺ still acts as crossing bridge in the prepared process. Figure 1.1 shows the tensile of hydrogel by measuring its crosslinking density, data suggested that as the Ca²⁺ concentration increased, crosslinking of alginate hydrogel increased that formed tighter construction.

### Swelling ability comparison between three kinds of alginate hydrogel

Hydrogel was utilized in extensive ways on medication because its three dimensional structure formed by polymer chains that presents soft and flexible characteristics similar to natural tissue in swelling condition. Thus, well hydrogels have several critical abilities to maintain three dimensional networks and water retensiveness in utilization. However, the tighter the 3-D networks, the poor the swellability of hydrogel, but the looser the structure, the poor the maintenance. According to previous study, Ca²⁺ are core factor affects the network of hydrogel, therefore, calcium alginate hydrogels with higher Ca²⁺ linkage have poor swellability(Fig 1.2 ), but not γ-PGA/alginate hydrogels.

**pH-sensitivity comparison between three kinds of alginate hydrogel**

In our previous work showed that alginate hydrogel is poorly water-soluble in low pH conditions and dissolvable in alkaline environments as a result of its acidic groups exhibit the alteration of COOH/COO⁻ thus the pH-sensitive crosslinked alginate hydrogels are currently designing for utilizing in drug delivery systems. Here we investigated pH-sensitivity of alginate hydrogels in different pH conditions, Fig 1.3 show that the pH-sensitivity of γ-PGA/alginate hydrogel is better than calcium alginate hydrogel, especially calcium formed γ-PGA/alginate hydrogel. Data suggested that Ca²⁺ crosslinking formed compact networks within alginate hydrogel lead to the failure of COOH/COO⁻ responsiveness, but with the existence of γ-PGA, hydrogel could retain its pH-sensitivity in wide pH changes.

**Drug release test**

According to above experiments, we assumed that γ-PGA/alginate hydrogels have better ability for utilizing in drug delivery. Thus we examined the drug release mechanism of γ-PGA/alginate hydrogels by loading ascorbic acid 2-glucoside(AA2G), a hydrophilic derivate from vitamin C. The measurement held when water molecular penetrated into the network by diffusion and the loaded AA2G diffused out into the media. In general, the releasing rate should be lower for gels with higher crosslinking density, data showed conformably in Fig. 1.4
This may result from the fact that for the chains are relaxed to a greater extent in the later part of drug-release process, thus causing a faster diffusion of the loaded AA2G into the medium, suggested great potential of $\gamma$-PGA/alginate hydrogels in drug delivery system.

**DISCUSSION**

Here are three kinds of alginate hydrogels compared with each other, alginate-calcium chloride hydrogel, alginate-sodium form polyglutamic acid hydrogel and alginate-calcium form polyglutamic acid hydrogel. Traditionally, alginate gel beads are commonly prepared in forms of sodium or potassium alginate solution into an aqueous solution of calcium ions typically from calcium chloride ($\text{CaCl}_2$) to make alginate-calcium chloride hydrogel. The gelation of $\text{CaCl}_2$ in varying crosslinking density and a polymer concentration gradient within the gel bead influenced the properties of alginate hydrogels, the amount and nature of sponged liquid substantially affected the porosity and mechanical strength of gel networks as well. Calcium alginate was recently been used as cell delivery vehicles for in vivo tissue engineering research and drug carrier that can be easily degraded by enzymes in the organism and eliminated from the body. A major disadvantage to the use of $\text{CaCl}_2$ is that gelation kinetics is difficult to control, and the resulting structure is not uniform, the rate of degradation of typical alginate hydrogels varies. It is improved by exploiting of joined polyglutamic acid with sodium alginate solution or calcium alginate, in this method, $\text{CaCl}_2$ was used as adjuvant instead. The network parameters including average molecular weight between crosslinks and the cross-linking density (ve), and value of compression modulus (G) of the alginate hydrogels, they were examined to evaluate the swelliability, pH sensitivity, fluids maintenance and drug release mechanism.

Data shows that increasing calcium content promoted the crosslinking density and decreased molecular weight between crosslinks in the alginate hydrogels. The increased crosslinking density leads to decreased swellability and flexibility and retentiveness of hydrogels result from the more compact network by calcium linkage. Copolymerized alginate-sodium form polyglutamic acid hydrogels and alginate-calcium form polyglutamic acid hydrogels overcome the defect because PGA has more hydrogen bonds of carboxyl group replaced calcium-formed ionic networks. The carboxyl group on the polymer chain residues of PGA present fine pH sensitivity and creates flexible, highly absorbing, smother hydrogels. Results of the drug-release mechanism and diffusion coefficients measurements of alginate hydrogels also indicated that the diffusion coefficients depend on not only the network structure of polymer matrix but also on the external environmental changes such as media pH, enzymes, and ionic strength, and pointed out that alginate hydrogels have great potential in drug delivery system. All the investigations of alginate hydrogels suggest that these hydrogels can lead to successful application for the medical and pharmaceutical utilization especially alginate-calcium form polyglutamic acid hydrogels.

**REFERENCES**


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