Fabrication of highly stretchable hydrogel based on crosslinking between alendronates functionalized poly-γ-glutamate and calcium cations

Masahiko Nakamoto [a], Moe Noguchi [a], Akihiro Nishiguchi [a], João F. Mano [b], Michiya Matsusaki [a,*], Mitsuru Akashi [a,c,**]

[a] Division of Applied Chemistry, Osaka University, Graduate School of Engineering, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Department of Chemistry, CICECO-Aveiro Institute of Materials, University of Aveiro, Campus Universitario de Santiago, 3810-193, Aveiro, Portugal
[c] Department of Frontier Biosciences, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka, 565-0871, Japan

ABSTRACT

We report a highly stretchable hydrogel based on the crosslinking structure between calcium cations and alendronates (ALN) conjugated with poly-γ-glutamate (γ-PGA), a typical biodegradable polymer. γ-PGA with ALN (γ-PGA-ALN) forms the hydrogel in the aqueous solution containing $CaCl_2$. The hydrogel shows 2000% of stretchability and reversible stretching without failure at a strain of 250%. The fracture strain and stress are tunable by varying the concentration of either γ-PGA-ALN or $CaCl_2$, indicating the importance of fine-tuning of the density of the cross-linkage to control the mechanical properties of the hydrogel. We believe the biodegradable polymer based highly stretchable hydrogel has potential for use in various fields such as tissue engineering.

1. Introduction

Biodegradable polymers are of great interest in the fields of tissue engineering and/or drug delivery [1]. Mechanical properties such as strength, stiffness, toughness or stretchability are often some of the most important functions in the application of these materials [2]. Mechanobiological studies have revealed that the mechanical properties of living constructs such as the extracellular matrix (ECM) strongly affect various biological processes from the molecular to tissue level, including tissue morphogenesis, cancer progression and tissue re-modeling [3] as well as ensuring the mechanical stability and structural integrity of tissues and organs [4]. Regulation of the mechanical properties of ECM often relies on the non-covalent cross-linkages including hydrogen bonding, electrostatic interaction and/or hydrophobic interactions in addition to the covalent crosslinked structure [5,6]. Non-covalent crosslinking allows reversible association/dissociation, so it can exchange the crosslinking points upon mechanical stress, enabling the high and reversible stretchability of ECM [7,8].

The introduction of non-covalent crosslinking in a polymer is also an effective strategy for enabling the control of the mechanical property of a synthetic hydrogel. A supramolecular host-guest recognition [9], [-11]

metal coordination [12], electrostatic interaction [13], hydrophobic interaction [15], and/or hydrogen bonding [14] have been introduced in synthetic hydrogels as non-covalent crosslinkers. Calcium ions are essential for the construction of inorganic-organic hybrid materials for various biological functions such as protection and/or mechanical support in living systems. These are often three-dimensional macromolecular assemblies of proteins, polysaccharides, and/or glycoproteins [16]. In the fabrication of hydrogel materials, it has been reported that the alginate forms a hydrogel by the crosslinking structure with calcium cations, known as the egg box junction [17]. Suo et al. reported that an alginate-calcium hydrogel with an interpenetrating network consisting of crosslinked polyacrylamide showed remarkable stretchability and toughness [18–20]. Taking inspiration from biomineralization, Cölfen et al. reported a highly stretchable hydrogel consisting of amorphous calcium carbonate and poly-acrylic acid, a material they named 'mineral plastic' [21]. Bisphosphonates such as alendronate (ALN) are known to have a high affinity with calcium cations, and have been used medicinally for osteoporosis [22,23]. In addition to its use as a small molecule drug, the conjugation of ALN with a polymer has been considered to be a promising strategy for engineering a bone-targeting carrier for drug delivery, regeneration therapy and/or tissue engineering [24–26]. Since

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* Corresponding author.
** Corresponding author.
E-mail address: m-matsus@chem.eng.osaka-u.ac.jp (M. Matsusaki).

ALN forms a 2:1 complex with a calcium cation [27], the interaction between ALNs and calcium cation can function as a non-covalent cross-linker in a hydrogel network. It has been reported that hydrogels consisting of polymers with ALN showed self-healing properties due to the dynamic nature of the cross-linkage. [28,29].

However, little has been reported on the fabrication of highly stretchable hydrogels based on the crosslinking between a biodegradable polymer functionalized with ALN and calcium cations. In this study, we demonstrate a highly stretchable hydrogel with tunable mechanical properties consisting of poly-γ-glutamate functionalized with ALN (γ-PGA) and calcium cations. γ-PGA is a naturally occurring polymer secreted by a *Bacillus subtilis strain*, and provides a potential resource for environmental and biodegradable materials [30]. We previously reported γ-PGA hydrogels crosslinked with disulfide bonds for the construction of three-dimensional engineered tissues by collapsing the hydrogels in response to a reduction condition [31,32]. ALN conjugated γ-PGA is expected to form crosslinking structure in response to calcium cations for the provision of a biodegradable hydrogel. We believe the highly stretchable hydrogel with tunable mechanical properties consisting of the biodegradable polymer would be a promising material as a scaffold in tissue engineering for bone regeneration.

2. Materials and methods

2.1. Materials

All the chemicals were used without further purification. Poly-γ-glutamic acid (γ-PGA, Mw = 200–500 kDa) and sodium hydrogen carbonate (NaHCO₃) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride *n*-hydrate (DMT-MM) was purchased from Watanabe Chemical Ind., Ltd. (Hiroshima, Japan). Alendronic acid (ALN) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The dialysis membrane (MWCO 15 kDa) was purchased from Spectrum Laboratories, Inc. (CA, USA).

2.2. Preparation of γ-PGA-ALN

One gram of γ-PGA was dissolved in 100 mL of 200 mM NaHCO₃ (aq.), followed by the addition of 2.1 g of DMT-MM and 1.9 g ALN. The solution was stirred at room temperature for 24 h. γ-PGA-ALN with 11 mol% or 37 mol% ALNs was prepared by tuning the reaction pH to 6.4 or 8.4, respectively. After the removal of the solvent by evaporation and vacuum, the polymer was purified by precipitation in ethanol and dialysis against water (MWCO: 15 kDa). The obtained γ-PGA-ALN was freeze-dried and analyzed by ¹H NMR, ³¹P NMR and elemental analysis.

2.3. Preparation of the γ-PGA-ALN gels

Hundred μL of γ-PGA-ALN was added into 1.5 mL of water containing 1 M CaCl₂ and the mixture solution was stirred at room temperature for 10 min. After the formation of the white hydrogel, it was molded using a silicon sheet (14mm × 4mm × 2 mm) and glass plates.

2.4. Tensile stress-strain measurement

The tensile stress–strain measurements were performed using a tensile tester at a deformation rate of 20 mm min^{-1} in air at room temperature. The sample length, width and thickness were 14 mm, 4 mm and 2 mm, respectively.

2.5. Evaluation of the absorption amount of calcium cations by the hydrogels

The amount of calcium cations in the hydrogel was quantified by the colorimetric titration method [39]. 100 μL of 30 wt% γ-PGA-ALN was added into 1.5 mL of water containing various concentrations of CaCl₂ (0.1, 0.2, 0.5, 1, 2 or 4 M) and stirred for 10 min. The influence of the incubation period was evaluated at a fixed CaCl₂ concentration (1 M). After gelation, the hydrogel surface was gently wiped and dried by lyophilization. The dried sample was dissolved in MilliQ by continuous stirring. The pH of the obtained solution was tuned to 13 by adding 8 M potassium hydroxide. Next, 2-hydroxy-1-(2-hydroxyl-4-sulfo-1-naphthy-lazo)-3-naphthoicacid (NN indicator) was added into the solution until the color of the solution changed to red-purple. Then, the titrant, 0.01 M ethylenediaminetetraacetic acid (EDTA) was added to the solution. The endpoint of the titration was defined as the volume of EDTA required to obtain a colorimetric change of the NN indicator from dark purple to blue.

2.6. Statistical analysis

All data are expressed as the means ± SD, unless otherwise specified. The values represent the means ± SD from three independent experiments. Statistical comparisons between groups were analyzed using two-tailed Student's T-tests. A *p* value ** = <0.05 and * = <0.01 was considered statistically significant.

3. Results and discussion

3.1. Preparation of γ-PGA-ALN gels

ALN was introduced in γ-PGA, a typical biodegradable polymer [30–33] by amide coupling reaction using (4-4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMT-MM) as a coupling reagent (Fig. 1a) [34]. The obtained γ-PGA-ALN was characterized by ¹H NMR, ³¹P NMR and FT-IR (Figs. S1 and S2). The incorporation amount of ALNs in γ-PGA was determined to be 11% from the elemental analysis. To validate the capability of γ-PGA-ALN to form a hydrogel responding to calcium cations, 100 μL of 10 wt% γ-PGA-ALN was added into 1.5 mL of water containing 1 M CaCl₂ and incubated at room temperature (Fig. 1b). A transparent hydrogel immediately appeared upon the addition of γ-PGA-ALN into the CaCl₂ solution, and then the hydrogel turned white within 10 min (γ-PGA-ALN in Fig. 1c). Here, we confirmed that γ-PGA without ALN showed no gelation in the CaCl₂ solution (γ-PGA in Fig. 1c). In addition, gelation was not observed when γ-PGA-ALN was added into the solution containing mono-cations such as lithium, sodium and potassium due to the weak affinity for ALN. γ-PGA-ALN formed a fragile film on the solution surface in response to MgCl₂, whereas γ-PGA-ALN formed rigid gels in response to BaCl₂ and CuCl₂ (Fig. 1d). Since these divalent cations have different size [35], these results may indicate that the size of di-valent cations affects the capability to form the stretchable polymer network with γ-PGA-ALN. We concluded from these results that ALNs in γ-PGA form crosslinking structures with calcium cations by selective interaction (Fig. 1e) [28,29]. The stress-strain curve measurement of the molded γ-PGA-ALN gel revealed the high stretchability of the hydrogel: the fracture strain and stress were determined to be 1900% and 23 kPa, respectively (Fig. 2a and b). The loading–unloading curve of the hydrogel shows a hysteresis loop, indicating the energy dissipation of the hydrogel (Fig. 2c). These results may indicate an extension of polymers from collapsed globules to a stretched coil structure upon tension, that is coupled with exchanges of the crosslinking points between ALN and calcium cations. The cyclic loading–unloading curve also confirmed a reversible stretch of the hydrogel without significant loss of stress at a strain of 250%. In addition, the hysteresis loop decreased with the increase of the number of the cycle from 1 to 3, and then reached constant for further cycles. This may arise from the rearrangement of the hydrogel network occurred during the cyclic test.
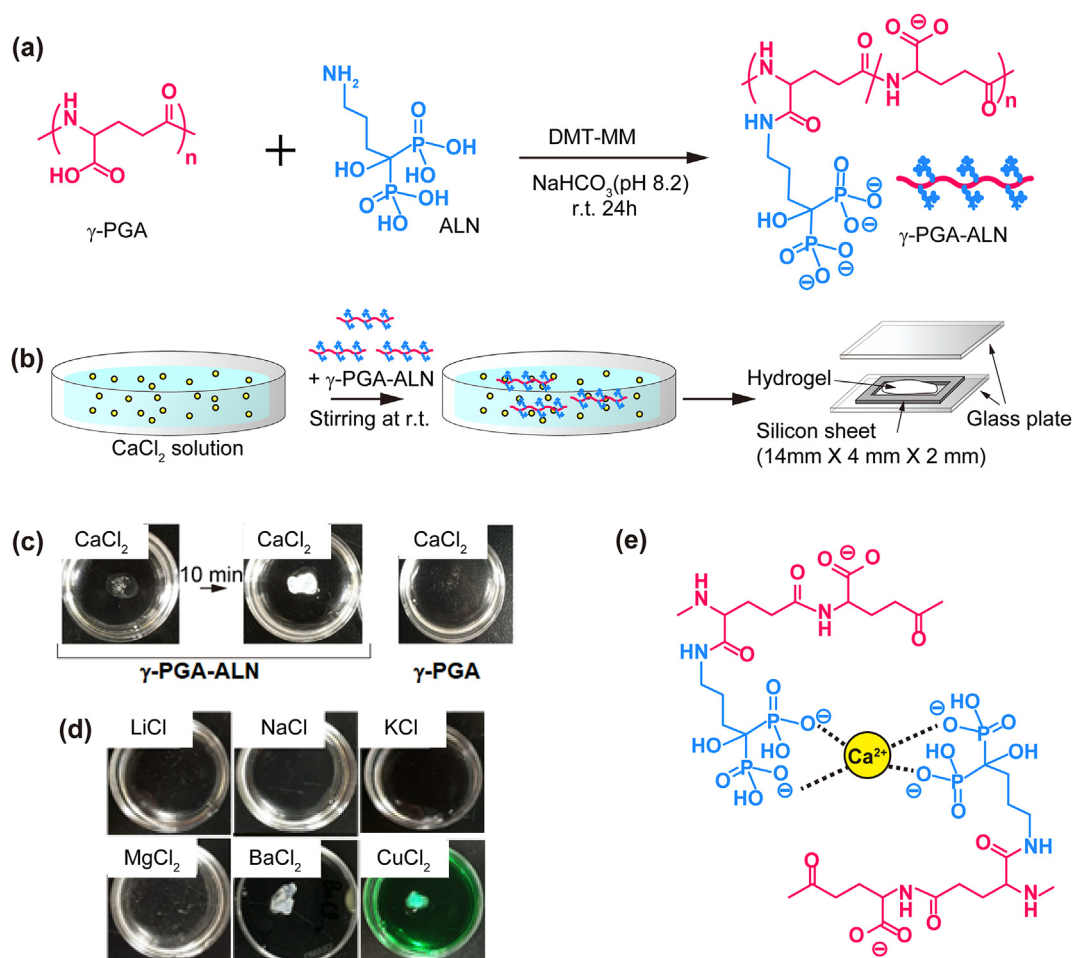
Fig. 1. Preparation of γ−PGA-ALN gels. (a) Synthesis of γ-PGA-ALN by amide coupling reaction. (b) Schematic illustration of the γ-PGA-ALN hydrogel preparation. (c) Photographs showing the formation of the hydrogel upon the addition of γ-PGA-ALN into an aqueous solution containing $CaCl_2$. The transparent pellet (left) became a white hydrogel (middle) within 10 min, whereas γ-PGA did not form a gel (right). (d) γ-PGA-ALN in the aqueous solution containing 1 M LiCl, NaCl, KCl, $CaCl_2$, $MgCl_2$, $BaCl_2$ or $CuCl_2$. (e) The proposed structure of crosslinking structure between ALNs and a calcium cation.
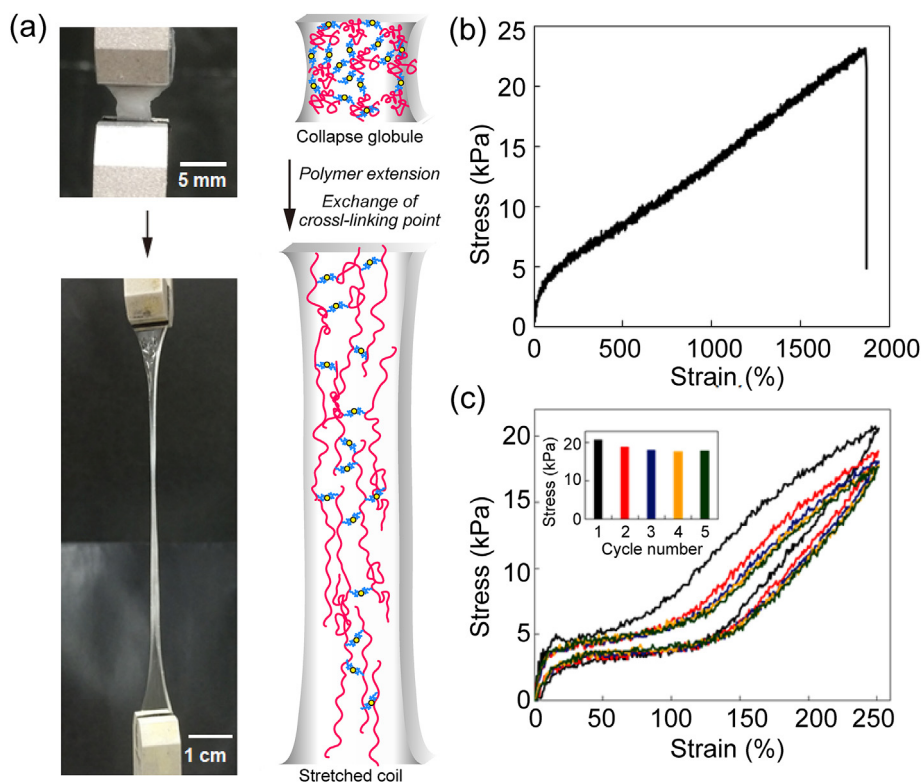
3.2. Effect of calcium ion and polymer concentrations on mechanical property of γ-PGA-ALN gels

Next, we prepared the γ-PGA-ALN gel with various polymer concentrations at a fixed $CaCl_2$ concentration (1 M) and incubation time (10 min) to demonstrate tunable mechanical properties of the hydrogel (Fig. 3a). The fracture stress increased with an increase in concentration of γ-PGA-ALN, whereas the fracture strain decreased due to the high density of crosslinking structure in the hydrogel. The influence of ALN incorporation amount in γ-PGA was also investigated using γ-PGA functionalized with 37% ALNs (Figs. S1 and S2). γ-PGA with 37% ALNs formed aggregation at lower calcium concentration than γ-PGA with 11% ALNs, whereas, obtained aggregation was rigid and no longer stretchable hydrogel (Fig. 3b). When ALNs are concentrated excessively in polymer networks, polymers would form excess crosslinked networks with calcium ions both intra- and inter-polymer, that fix the γ-PGA-ALN as a rigid globule polymer. We concluded from these results that the fine tuning of the density of the cross-linkage between ALN and calcium cations in a polymer network allows the control of the mechanical properties. We further investigated the influence of the $CaCl_2$ concentration and incubation period during the gelation on mechanical properties of the hydrogel consisting of γ-PGA with 11% ALNs at a fixed concentration of precursor polymer solution (30 wt%). The concentration of calcium cations during the gelation significantly affected the mechanical properties of hydrogels when the incubation time was fixed at 10 min: the fracture strain decreased from 3000%–200% and the fracture stress

increased from <10 kPa to 400 kPa with the increase of $CaCl_2$ concentration from 100 mM to 4 M (Fig. 3c). Although there was the trade-off relationship between stretchability and stress, a maximum stretchability of γ-PGA-ALN hydrogel (3000% of maximum strain) observed at lowest $CaCl_2$ concentration was higher than or comparable with that in earlier reports on highly stretchable hydrogels [18–20,36–38]. We concluded from this result that the tuning of the occupancy of ALNs with a calcium cation in the hydrogel allows the control of the mechanical properties of the biodegradable polymer based γ-PGA-ALN hydrogel across a wide range. On the other hand, the influence of the incubation period on the stress and strain was slight when the hydrogel was prepared in the presence of 1 M $CaCl_2$ (Fig. 3d). It may be more likely that the density of the cross-linkage between ALN and calcium cations is dominated by the equilibrium stability of the complexation.

3.3. Crosslinking mechanism of γ-PGA-ALN gels

The interaction between γ-PGA-ALN and calcium cations was assessed by the evaluation of the absorption amount of calcium cations by the hydrogel (Fig. 4). When the incubation time was fixed at 10 min, the absorption amount linearly increased with an increase in concentration of $CaCl_2$ from 0.1 to 2 M and decreased at 4 M (Fig. 4a). The decreased absorption at 4 M $CaCl_2$ may have arisen from the squeezing effect induced by the salting out. On the other hand, the effect of incubation time on the absorption was slight (Fig. 4b). These results are consistent with the arguments put forward earlier. Interestingly, the

Fig. 2. Stretching property of γ-PGA-ALN gels. (a) Photograph showing the high stretchability of the γ-PGA-ALN gel. Schematic illustration depicts the proposed conformational change of γ-PGA-ALNs in the hydrogel. (b) The stress-strain curve of the γ-PGA-ALN gel prepared from 10 wt% γ-PGA-ALN and 1 M CaCl$_2$. (c) The stress-strain curves for the reversible stretch of the γ-PGA-ALN hydrogel (10 wt%). The inserted figure shows the stress for each cycle.
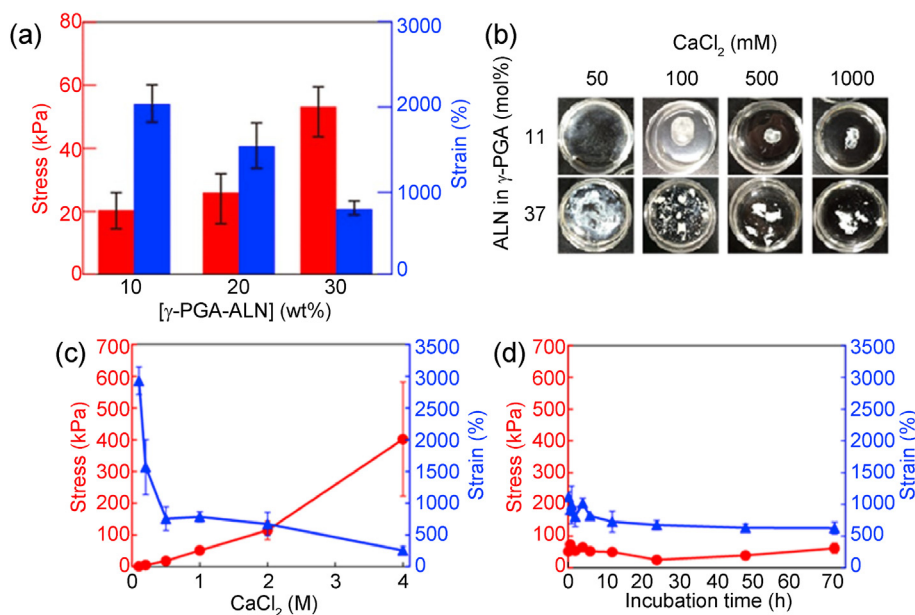
Fig. 3. Effect of calcium ion and polymer concentrations on mechanical property of γ-PGA-ALN gels. (a) The fracture stress and strain of the γ-PGA-ALN gel prepared from various concentrations of γ-PGA-ALN. The concentration of CaCl$_2$ was fixed at 1 M. (b) The effect of the population of ALN conjugated with γ-PGA on gelation. 30 wt% of γ-PGA-ALN was added into water containing various concentrations of CaCl$_2$ and incubated for 10 min. (c) The effect of CaCl$_2$ concentration on the stress and strain of the γ-PGA-ALN gel. The incubation time was fixed at 10 min. (d) The effect of the incubation time in the gelation process on the stress and strain of the γ-PGA-ALN gel. CaCl$_2$ concentration was fixed at 1 M.

calcium ions absorbed by the gel were five to ten times greater than the number of ALNs in the gel. This result indicates that remaining glutamate residues in the polymer function to retain excess calcium ions in the hydrogel by weak electrostatic interaction [23], perhaps, that provide calcium ions to the alendronates effectively upon the stretch as sacrificial bonds [9]. We further assessed the effect of the introduction of the additional covalent crosslinker, ethylene glycol diglycidyl ether (EGDGE) in the γ-PGA-ALN gel on the mechanical properties of the gel (Fig. 4c and

S3). The addition of EGDGE during the molding process resulted in the increased fracture stress and decreased fracture strain, respectively, due to the introduction of a permanent crosslinking structure (Fig. 4d). This result emphasizes the importance of the dynamic nature of the crosslinking between ALN and calcium cations for the high stretchability. This result also indicates the capability of the introduction of the permanent crosslinker to control the mechanical stability and property of γ-PGA-ALN hydrogel. Indeed, the stability of the hydrogel in PBS could
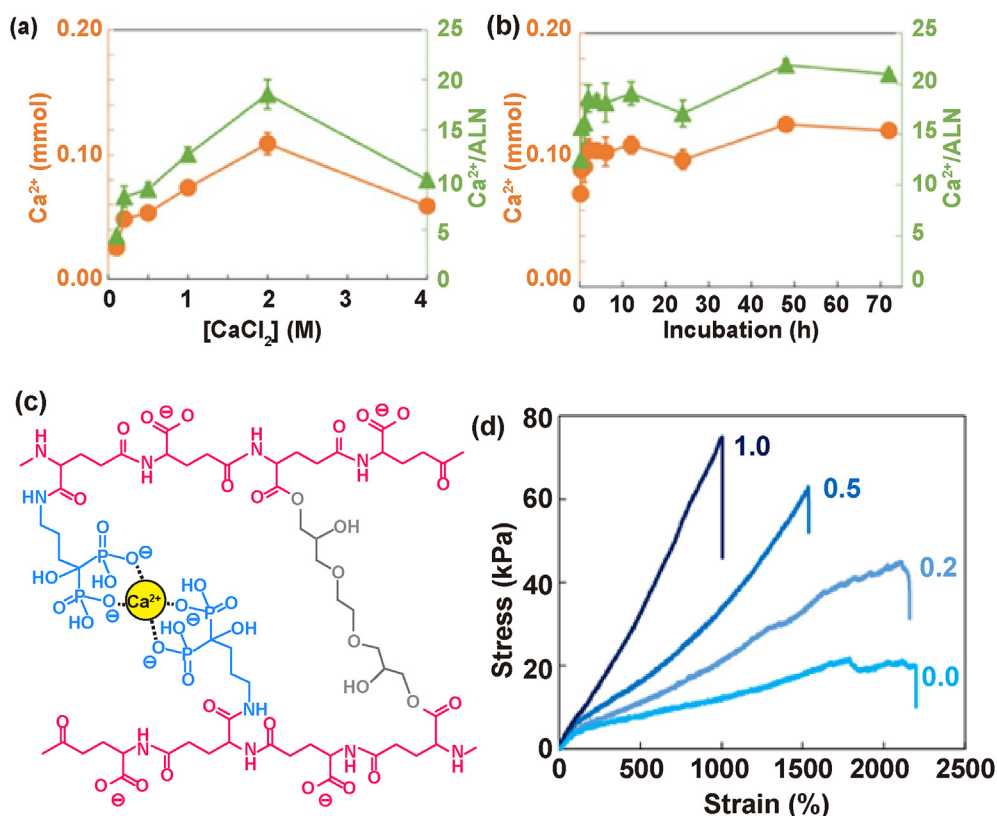
Fig. 4. Crosslinking mechanism of γ-PGA-ALN gels. (a) The effect of CaCl₂ concentration on the absorption of calcium cations by the γ-PGA-ALN gel. The incubation time was fixed at 10 min. (b) The effect of the incubation time on the absorption of calcium cations by the γ-PGA-ALN gel. CaCl₂ concentration was fixed at 1 M. (c) The chemical structure of γ-PGA-ALN covalently crosslinked by EGDGE. (d) The effect of covalent crosslinking on the mechanical properties of the γ-PGA-ALN gel. The weight ratio of EGDGE/γ-PGA-ALN is inserted in the figure. The concentration of γ-PGA-ALN was fixed at 10 wt%.

be prolonged by the introduction of EGDGE (Fig. S4). Lastly, we preliminary confirmed the adhesion and elongation of normal human dermal fibroblasts (NHDF) on γ-PGA-ALN hydrogel (Fig. S5), indicating its suitability as a scaffold for tissue engineering, although further investigations are currently ongoing.

4. Conclusions

In this work, we developed the highly stretchable hydrogel consisting of the biodegradable polymer by engineering the crosslinking structure between calcium cations and ALNs in a γ-PGA-ALN polymer network. γ-PGA-ALN formed a hydrogel in the presence of CaCl₂ and the obtained γ-PGA-ALN gel showed 2000% of stretchability and reversible stretching without failure at a strain of 250%. The mechanical properties of the γ-PGA-ALN gel could be controlled by tuning the concentration of γ-PGA-ALN or calcium cations in addition to the introduction of the permanent cross linker. Since NHDF adhered on/in γ-PGA-ALN gel by mixing gelation and subsequent culture, we believe the biodegradable polymer-based hydrogel with tunable mechanical properties reported in this study has the potential to be used as a scaffold in tissue engineering for bone regeneration.

Author statement

M.M. and M.A. contributed to conceptualization, methodology, writing, supervision, reviewing and editing, project administration and funding acquisition. M.N. and A.N. contributed to methodology, validation, investigation, and writing the original draft. J.F.M. contributed to validation and supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2022.100225.

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