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Transglutaminase crosslinked sodium caseinate/starch/tri calcium phosphate based flexible sponge grafts

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ABSTRACT

In this study, sodium caseinate/starch/ tri-calcium phosphate (TCP) base scaffolds were fabricated and cross-linked with microbial transglutaminase. A Maillard reaction was carried out to stabilize the blend of sodium caseinate (8% w/w) and starch (8% w/w) solutions. The dynamics of enzymatic cross-linking activity were assessed with different TCP concentrations. Freeze-dry method was used to extract water from the gel system to acquire a porous, spongy structure. Apart from many other enzymes, microbial transglutaminase has the capability to be active even below freezing temperatures. Keeping at −50 °C for 12 h, enzymes were still active to complete the post-crosslinking process. 0.5% TCP case resulted in 690 kPa ultimate tensile strength at dried state. Cell activity and characterization tests were elucidated and observed that those scaffolds can have very promising applications in bone tissue engineering.

1. Introduction

3D biological scaffolds can be intended for filling bone gaps and voids in bone healing as flexible and formable substances [1]. Structures that imitate extra-cellular matrix by obtaining from natural polymers and hydrogels can be obtained with different fabrication techniques [2]. These scaffolds have stabilization problems which are surmounted by usual post-processing with toxic solvents or expensive crosslinkers [3].

On the other hand, the green and cost-effective method of enzymatic cross-linking of 3D scaffolds raises various difficulties like weak bonding. Microbial transglutaminase (mTG) forms a covalent bond between primary amines and glutamine residues and creates a gel structure when it is injected into the precursor solution. If this enzyme, also known as meat glue, is not inhibited or removed from the system afterward, it continues to cross-link with a slower diffusion rate.

Water-soluble protein-based scaffolds are subjected to post-crosslinking after removal from the freeze-drier from which the ice crystals are extracted and porous scaffolds are created. If the crosslinker is mTG, the scaffolds need to be placed in a water-based liquid medium, which means that the scaffold will rapidly degrade. Enzyme activity and mobility can be increased by keeping the scaffolds in a humid environment, where the rate of degradation (due to hitting water molecules on the surface of the scaffolds) does not exceed the rate of cross-linking. For this, sodium caseinate (NaC), one of the natural polymers, was first bonded with starch by the Maillard reaction. Tri-calcium phosphate (TCP) was incorporated in it to increase osteoconductivity. TCP added (see Supp. Table 1), starch bonded NaCs are further linked to create giant networks with enzyme. Post-crosslinked spongy scaffolds were kept in a saturated environment to end up with durable scaffolds. The physical and chemical properties of the scaffolds were characterized by electron microscopy analysis, mechanical, calorimetric, and degradation tests. The activities of osteoblast cells were measured via a cell viability test.

2. Materials and methods

The precursor solution was prepared by adding 8% wt/wt NaC and 8% wt/wt starch in distilled water at 70 °C. Solution was mixed slowly at below 60 rpm for 5 h at 70 °C inside a sealed glass. TCP's were added at four different ratios of 0, 0.25, 0.5, and 1% wt/wt inside the conjugated solution (Supp. Table 1). Final solutions were prepared by the addition of 4% wt/wt mTG inside the TCP and TCP-free cases at 40 °C.

The prepared hydrogels were then gently poured inside the 3D printed cylindrical molds (10x40 mm), and rectangular molds (10x45x5 mm) to test their compressive and tensile mechanical performances, respectively followed by a full gelation process for 12 h at 40 °C. Samples were transferred to −20 °C prior to freeze-dry for 2 h. The freeze-drying process was completed at −50 °C for 12 h. Samples were post-processed by keeping them at 40 °C for 48 h in a water vapor saturated closed chamber. Finally, samples were heat-treated at 85 °C for

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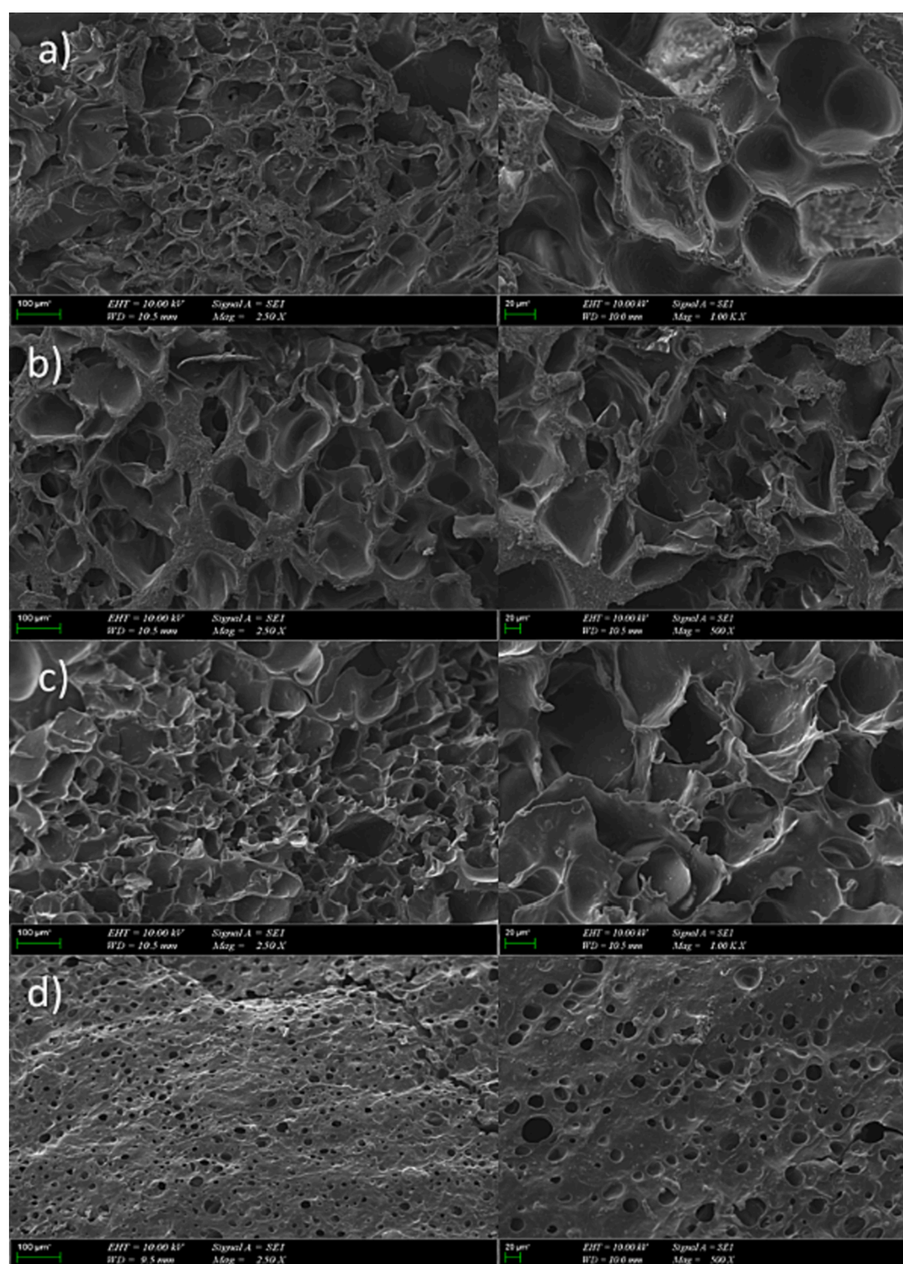

Fig. 1. SEM micrographs of the samples after freeze dry and post-process of water saturated air at 250× and 500× a) TCP free case, b) T25, c) T50, d) T100. (Scale bar of left column is 100 µm and right column is 20 µm.).

15 min to inactivate the enzyme.

3. Results & discussion

Mixture of starch and NaC is normally solvable in water but its stability was so low that precipitation occurs soon after mixing was stopped. However, the conjugated (8%wt) NaC and (8%wt) starch by the Maillard reaction [4] stood for long periods without precipitation at + 4 °C. Increasing the concentration of TCP inside the solution had raised another stability problem. Usage of large amounts of metal ions originating from TCP inside the solution lowered enzymatic activity even the source of the enzyme was microbial [5].

The color of the solution changed from yellow to brown with the mixing of NaC and starch. The color of the solution lightened again and turned into whitish with the addition of TCP. 4%wt enzyme cross-linked all groups in around 2 h. Samples were taken from the baker 10 min before full gelation, with the help of a syringe, and poured into molds made from 3D-ABS 100% infill which enabled homogenous specimens. The samples, which realized full gelation by waiting for 12 h in the mold, were shrunk by 15% after being placed in the freeze drier (Supp. Fig. 1). Shrinkage helped to remove from the mold easily. Samples were put at −20 °C after 12 h due to the fact that as the degree of crosslink increases, the pore-forming ability of the freeze-drying technique decreases which results in not-interconnected, superficial pores.

The surface morphology of T100 case was different from all other groups (Fig. 1d) with a surface porosity of 15 ± 5%. T0, T25, and T50 had porosities close to each other (57 ± 3, 51 ± 9, 46 ± 2%) and T25 had the thickest walls (16 ± 10 µm) and T50 had the thinnest walls (5 ± 1 µm). In T100, the pores were distributed in the form of wells due to weak crosslinking. After the freeze-drying step, samples were exposed to moisture for 48 h, melting had occurred on the upper surface of T100 with the water vapor. The water-soluble parts collapsed the surface pores. Lack of further cross-linking of T100 can be observed in other analyses. In mechanical tests (Supp Table1), the lowest tensile stress and

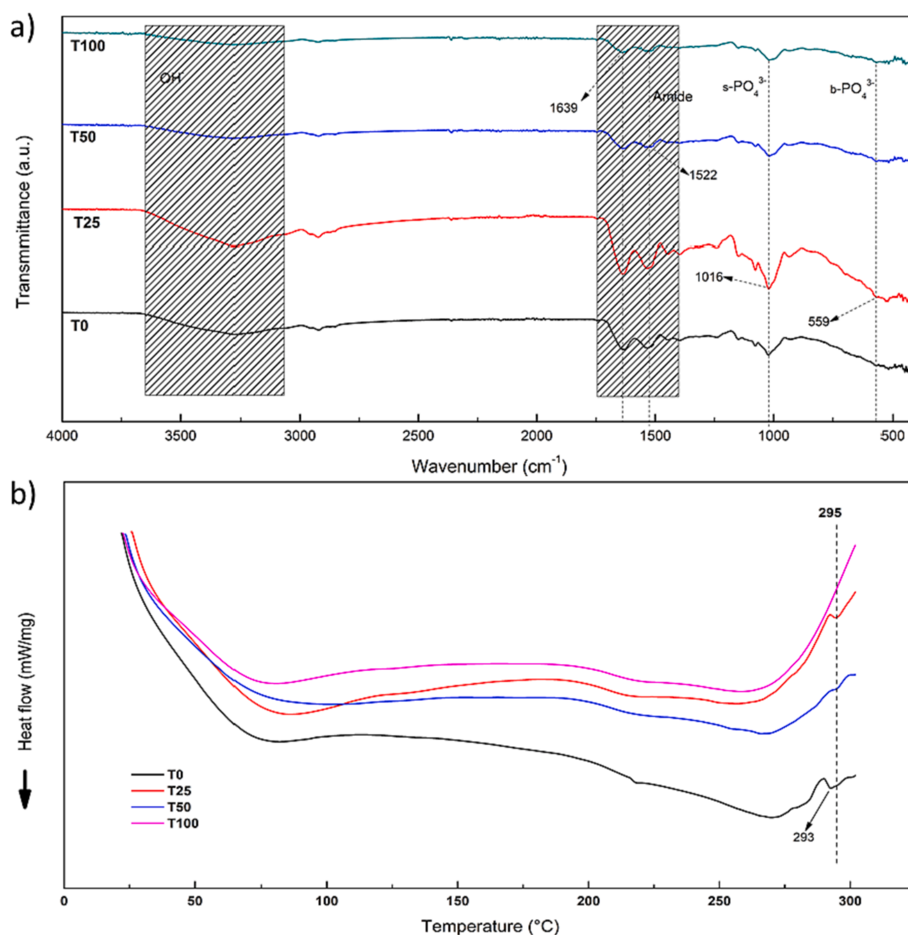

Fig. 2. a) Fourier Transformed IR analysis, b) DSC graphs of the samples in the scaffold form. (See Supp Fig. 4. for powder forms).

the lowest strain were T100 which supports SEM micrographs. Fig. 2(a) shows the FT-IR spectra of all samples and raw materials. The characteristic peak around 3250 cm^{-1} was attributed to the stretching mode of hydroxyl group (OH^-). The peaks at the range of 559 and 1016 cm^{-1} can be assigned to the bending and stretching mode of phosphate group ($PO4^{3-}$), respectively [6]. Positions of the Amide peaks revealed the presence of the NaC inside the scaffolds.

With the addition of TCP, shown in Fig. 2(b), the melting point specific to starch (282 °C) shifted to the right, while the peak specific to the NaC (269 °C) shifted to lower degrees for the composite scaffolds. In the case of T100, two peaks merged to a peak at 259. Gelatinization and the evaporation of the water inside the pure starch occurred around 100 °C which was suppressed by other constituents and did not observe inside the conjugated groups. Instead, denaturation of the protein structure was observed around 76–85 °C.

Collapse of the pore walls was easier when TCP was not used in the samples considering the flat part of the compression test as shown in Fig. 3a. “Three distinctive steps of porous scaffolds” were observed in the plots of selected states from each group (Fig. 3a): In the first stage, (i) elastic buckling of the walls occurred (ε: 0–13%), (ii) pore walls collapsed (plateau, ε: 13–50%), and finally (iii), the internal structure of the material was permanently destroyed [7].

The ultimate tensile strengths of the triplicate samples for each group were between 200 and 600 kPa (Fig. 3b). T25 was almost same as T50, and they were more ductile than T0. T100, on the other hand, had the lowest ductility(0,93%) and ultimate tensile strength(150 kPa). In PBS degradation measurements, all groups except T100 lost 70% of their weights on Day 2 (Fig. 3c). T100 had a sharper degradation curve. Degradation of not- post-crosslinked samples inside the aqueous environment occurred immediately after soaking them inside the water (Supp. Fig. 3). However, since alamarBlue® tests were in DMEM medium, all of the post-processed cases preserved their integrity even on the 7th day (Supp. Fig. 3).

An excess of the reduction percentage of alamarBlue is an indicator of metabolic activity, that is, high viability. All of the groups have a reduction of ~15% on the 7th day of the experiment except for the control group (~30%) as shown in Fig. 3d. The fact that the control group had twice viability wrt to other cases is related to the cell penetration into the 3-dimensional structure which is called as a 3D effect [8]. In the control group, cells were seeded on top of the two-dimensional well-plate surface which does not exhibit any out-of-plane migration.

4. Conclusion

Adding TCP below 1% revealed favorable results for bone scaffolds. The presence of cell activity in all groups indicated that NaC and starch can be used in soft scaffolds. The proposed conjugation technique enables the combination of both biomaterials’ superior properties in terms of availability and water-formability. Using enzyme for crosslinking added a greener way of fabricating scaffolds. T25 and T50 have better mechanical performances and morphologies wrt to other groups. The T0 case, where no TCP was used, was not different from the other two groups, but it is well known that TCP also provides good osseointegration, osseomigration, and bone regeneration characteristics. Therefore, it can be said that T25 and T50 have great potential as flexible scaffolds.

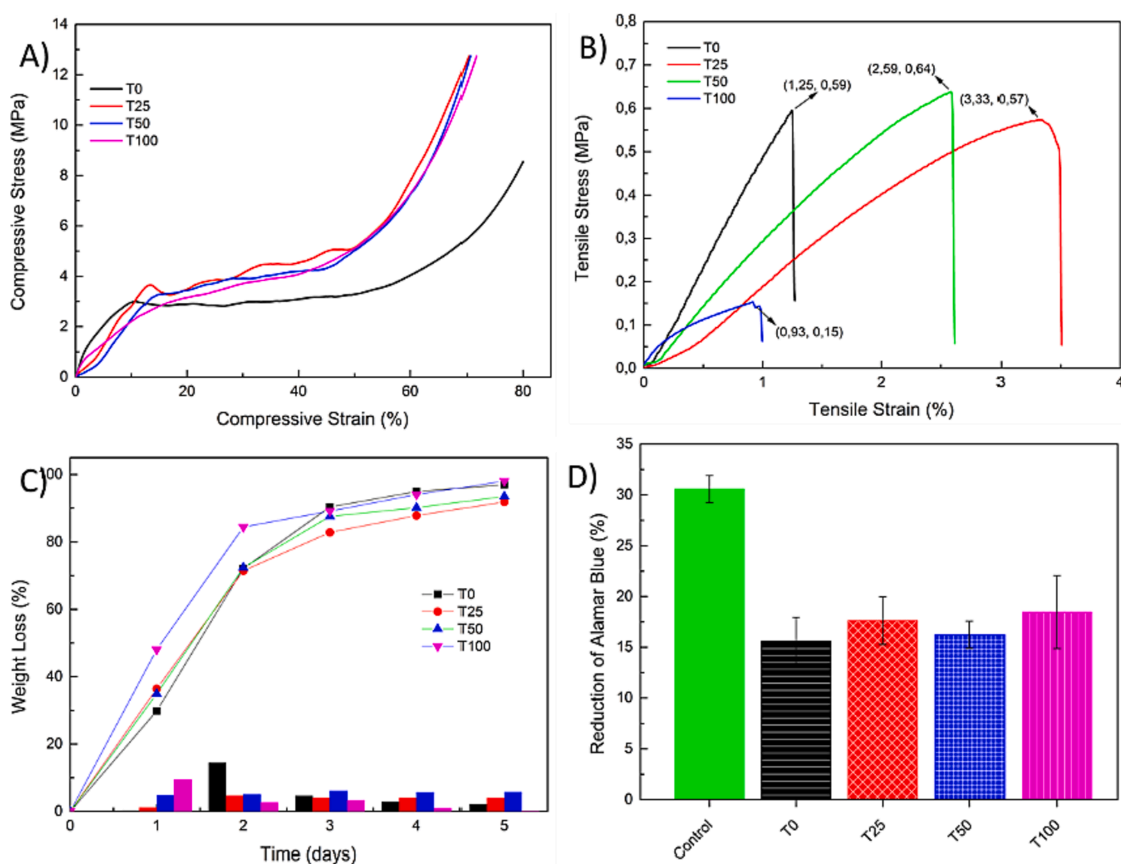

Fig. 3. A) Compression test graph B) Tensile Test graph C) Degradation profiles in PBS (Bars demonstrate standard deviations) D) Cell viability assay results of the groups.

CRediT authorship contribution statement

Mustafa Sengor: Conceptualization, Methodology, Software, Data curation, Writing – original draft, Visualization, Investigation, Supervision, Software, Validation, Writing – review & editing.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.matlet.2022.132943.

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