ORIGINAL ARTICLE

Effect of Dehydrothermal Treatment Conditions on Epigallocatechin Gallate-conjugated Gelatin Materials

Takahiro ENDO[1], Masato NAKAGAWA[2], Tomonari TANAKA[3], Yasuhiko MATSUSHIMA[2], Yoshitomo HONDA[2], and Shunsuke BABA[4]

[1]Graduate School of Dentistry, Department of Oral Implantology,
[2]Department of Oral Anatomy, Osaka Dental University, Osaka, Japan
[3]Department of Biobased Materials Science, Kyoto Institute of Technology, Kyoto, Japan
[4]Department of Oral Implantology, Osaka Dental University, Osaka, Japan

Synopsis
Gelatin-based materials are promising biomaterials for applications in medicine and dentistry. Dehydrothermal treated (vacuum heated) epigallocatechin-conjugated gelatin sponges with or without beta-tricalcium phosphate (β-TCP) granules show greater bone-forming ability than those without this treatment. However, there is a paucity of information associated with the effect of vacuum heating conditions for these materials. In this study, we verified the changes in temperature and time during vacuum heating for the surface topography and viscoelasticity of epigallocatechin-conjugated gelatin sponges with or without β-TCP granules. In scanning electron microscopic observation, there were negligible changes in microstructure or surface topography of epigallocatechin-conjugated gelatin sponges up to 200°C for 4 h or 150°C for 24 h. Meanwhile, in the dynamic viscoelasticity test, the values of the storage and loss moduli remarkably increased beyond 100°C after 4 h heating or extending the heating time up to 16 h at 150°C. This increase was presumably associated with cross-linking in the epigallocatechin-conjugated gelatin. These results offer insights into the use of dehydrothermal treatment with vacuum heating for gelatin-based biomaterials with polyphenols.

Key words*: gelatin, EGCG, dehydrothermal treatment, vacuum heating, viscoelastic tests*

Introduction

Gelatin, a fibrous macromolecular protein composed of denatured collagen, has been widely investigated for medical applications [1]. This denatured macromolecule is commercially extracted from the skin, bone, and cartilage of poultry (porcine, bovine, and chicken) and aquatic animals [2]. Each gelatin has different thermal (melting and setting points) and rheological (viscosity) properties at different temperatures [3] and is conventionally extracted using acidic and alkaline solutions [4]. Different extraction methods can give rise to different gelatines with distinct isoelectric points [4], which can change the adsorption behavior of proteins or small molecules when used as drug carriers [4]. In addition, chemical crosslinking procedures using condensing agents, such as EDC-NHS, or physical crosslinking using radiation, heating, etc. [2], have been used to improve the weak mechanical properties.

The cross-links can reinforce the mechanical properties of gelatins, such as the elastic modulus and viscoelasticity, and change their formability [1, 5]. When gelatin-based materials are applied to tissue engineering, the various

sizes of the interconnected porous structures affect tissue or cell invasion into these scaffolds [6, 7]. These pores in gelatin can be formed regularly by changing the freezing process, such as freezing temperature and air permeability of the mold before freeze-drying [7, 8].

Besides the above properties, the gelatin has many advantages, such as high biocompatibility, solubility, cellular attachment motif, low cost [1], and other excellent properties for its use as a biomaterial. Therefore, gelatin-based materials have been widely investigated as medical devices, such as cell scaffolds [9] and hemostatic agents [10], and occasionally used with substances, such as epigallocatechin gallate (EGCG) [11] and growth factors [4]. When used in regenerative medicine, although cross-linked gelatin is superior to collagen in terms of angiogenesis capacity [12], the ideal gelatin structure for tissue regeneration, for example osteoconductivity [6], is still under investigation.

EGCG, a catechin derived from green tea, exhibits multiple functions including anti-inflammatory [13], anti-oxidant [14], anticancer [15], and antibacterial activities [14]. Recently, we have developed an EGCG-chemically conjugated gelatin sponge (Ec-GS) with or without dehydrothermal treatment using vacuum heating [11, 16] and with porous β-tricalcium phosphate (β-TCP) granules [17] and have investigated its applicability as a bone substitute material [11, 16, 17]. When seeded with adipose-derived pluripotent progenitor cells, dehydrothermal treated (vacuum-heated) Ec-GS can induce great bone regeneration [9]. Additionally, when used with senescent dedifferentiated fat cells, vacuum-heated Ec-GS with β-TCP granules (vhEc-GS-β) could restore the osteogenic potential of the aging cells, partially by modulating the senescence-associated secretory phenotype (SASP) factors secreted from senescent cells [18].

Vacuum-heated (dehydrothermal treated) Ec-GS shows superior bone-forming ability than non vacuum-heated Ec-GS [16], while, in general, osteogenic or bone-forming abilities are the complex phenomena affected by diverse material properties, such as surface topography [19], pore size [20], porosity [21], and so forth. There is still a paucity of information about what material properties of Ec-GS and Ec-GS-β are affected by the dehydrothermal treatment. Given these backgrounds, this study was designed to verify the effects of changes in temperature or time during vacuum heat treatment on the microstructure, surface property, and viscoelasticity of Ec-GS with and without β-TCP granules.

Materials and methods

1. Preparation of Ec-GS and Ec-GS-β

The previously reported methods using aqueous chemical synthesis were applied to prepare Ec-GSs or Ec-GS-βs [17]. Both materials contained 0.28 w/w% EGCG, relative to gelatin [17]. Ec-GS-β containing porous β-TCP granules (500–1000 μm) were purchased from Cataly Medic Inc. (Chiba, Japan). The weight ratio between Ec-GS and β-TCP granules was approximately 1:4 in Ec-GS-βs.

2. X-ray diffraction and Fourier transform infrared spectroscopy

Powder XRD patterns were recorded using step scanning at 0.02 intervals from 5 to 60°, with Cu Kα X-rays on a diffractometer (XRD-6000; Shimadzu Co., Kyoto, Japan) at 40 kV and 30 mA. The attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectra of Ec-GS and Ec-GS-β were obtained using IRAffinity-1S (Shimadzu Co.) at a wavenumber of 500–2000 cm^{-1} at a resolution of 4 cm^{-1}.

3. Scanning electron microscopic analysis

Field-emission scanning electron microscopy (FE-SEM) (S-4800; Hitachi Ltd., Tokyo, Japan) was used to investigate the microstructure and surface topography of the samples. All samples were coated with OsO4 using an osmium coater (HPC-20 device; Vacuum Device, Ibaraki, Japan) before SEM observation. The surface microstructure of the samples was observed at a magnification of ×100, while the surface topography of the Ec-GS parts was analyzed at ×3,000 with an accelerating voltage of 5 kV.

4. Rheological measurements

The elastic moduli of the samples were measured using a rotational rheometer (Physica MCR 301; Anton Paar, Graz, Austria) with parallel plates of 8 mm diameter. Each sample was placed in the center of a Peltier plate, and 40 μL of ultrapure water was added using a micropipette. The plate-to-plate distance was set to 1 mm, and the plate was soaked in water for 1 min. Then, the elastic modulus spectra were recorded with the distance set to 150 μm while maintain-

ing a low deformation of 0.005 over a frequency range of 0.1–10 Hz at 20°C.

5. Statistics

All statistical analyses were performed using GraphPad Prism 7 J (GraphPad Software Inc., San Diego, CA, USA). Data were analyzed using one-way analysis of variance with the Tukey multiple comparison post-hoc test. Statistical significance was set at $p< 0.05$. Data are presented as the mean ± standard deviation (SD).

Result

1. Material evaluation

In X-ray diffraction measurements, the peaks of Ec-GS-β were similar to those of a single use of β-TCP granules (Fig. 1). There was negligible alteration in the β-TCP crystals or precipitation of other calcium phosphates during material synthesis (Fig. 1). Hereafter, No vh deems no vacuum heating. ATR-FTIR measurements revealed amide 1, 2, and 3 bond spectra (1633, 1538, and 1237 cm^{-1}, respectively) [22], which were possibly derived from the gelatin of Ec-GS in Ec-GS-β (Fig. 2). These results partially prove the presence of EGCG-chemically conjugated gelatin and β-TCPs (Figs. 1, 2).

2. Effect of temperature during vacuum heating treatment

Figure 3A shows SEM images of the samples with and without vacuum heat treatment at different temperatures. The images after treatment at 200°C were representatively shown because there were no remarkable changes from room temperature to 200°C. The plate or fibrous structure possibly associated with the EGCG-conjugated gelatin part can be visible in both images of Ec-GS and Ec-GS-β regardless of vacuum heating treatment. All samples formed heterogeneous surfaces with pore structures (Fig. 3A). Figure 3B shows images of the surfaces of the EGCG-conjugated gelatin parts of the samples with or without vacuum heat treatment. As with the smooth surface of the Ec-GS samples without vacuum heating treatment (No vh), there was no significant change in the surface topography after the temperature was increased up to 200°C (Fig. 3B). Using dynamic viscoelastic tests, we evaluated the effect of

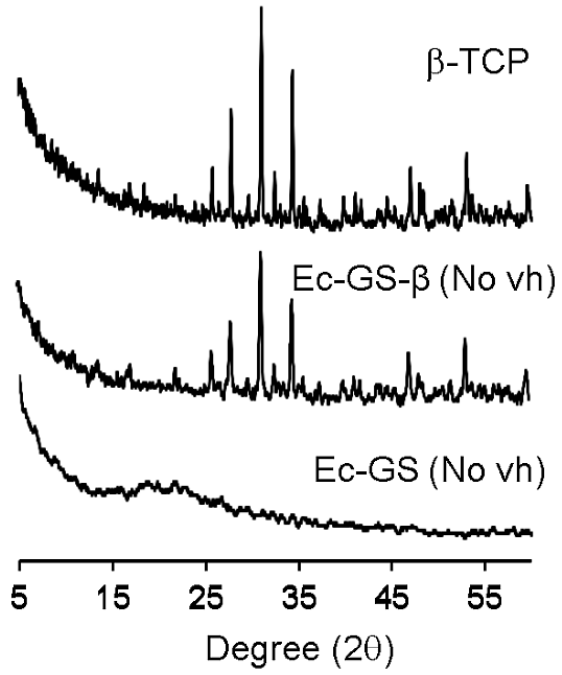

Fig. 1 X-ray diffraction pattern of the samples. β-TCP: beta-tricalcium phosphate granules. Ec-GS: epigallocatechin-conjugated gelatin sponges; Ec-GS-β: Ec-GS with β-TCP granules. No vh: no vacuum heating.

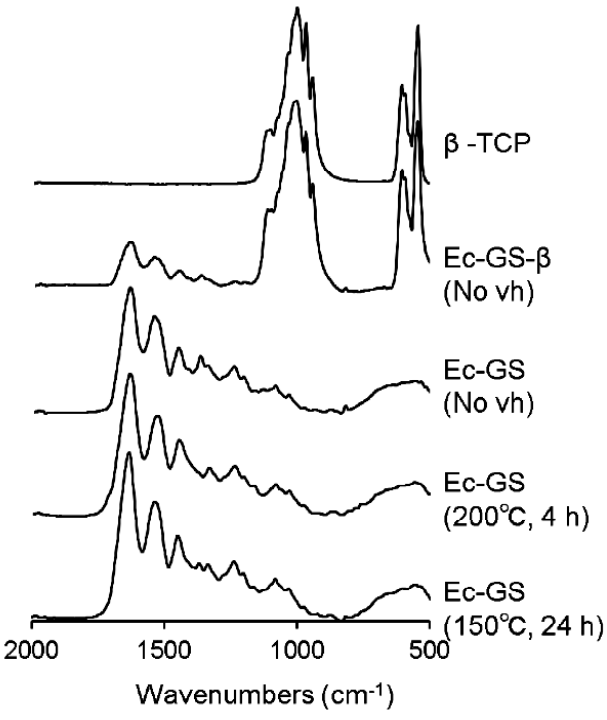

Fig. 2 Attenuated Total Reflection Fourier Transform Infrared Spectroscopy for the samples treated with or without vacuum heating (dehydrothermal treatment). Ec-GS: epigallocatechin-conjugated gelatin sponges; Ec-GS-β: Ec-GS with β-TCP granules. No vh: no vacuum heating.

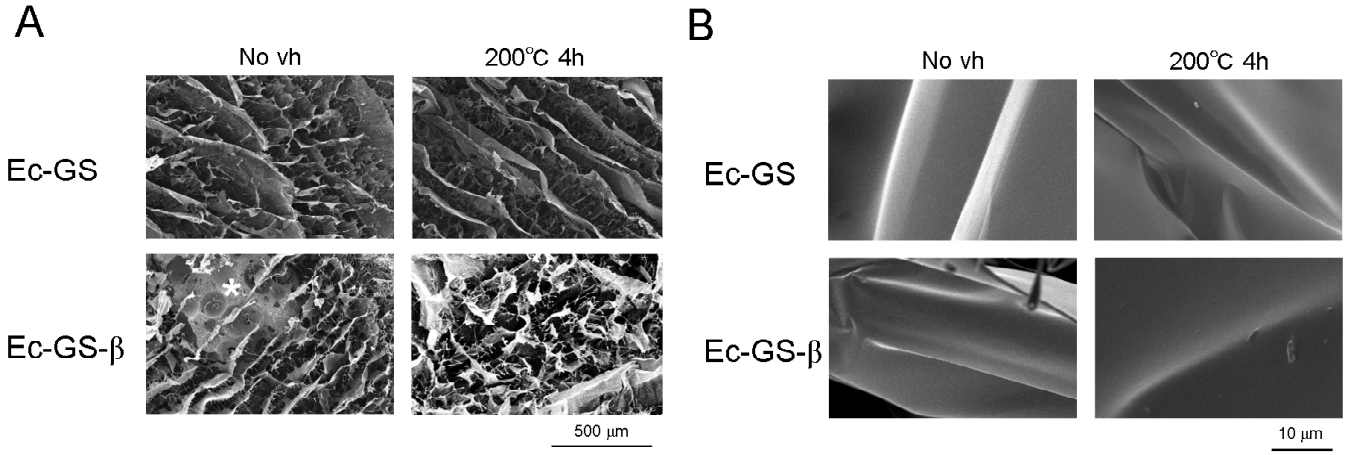

Fig. 3 Representative scanning electron microscopic images of samples with or without vacuum heating (dehydrothermal treatment). No vh: no vacuum heating. *: β-TCP granules.

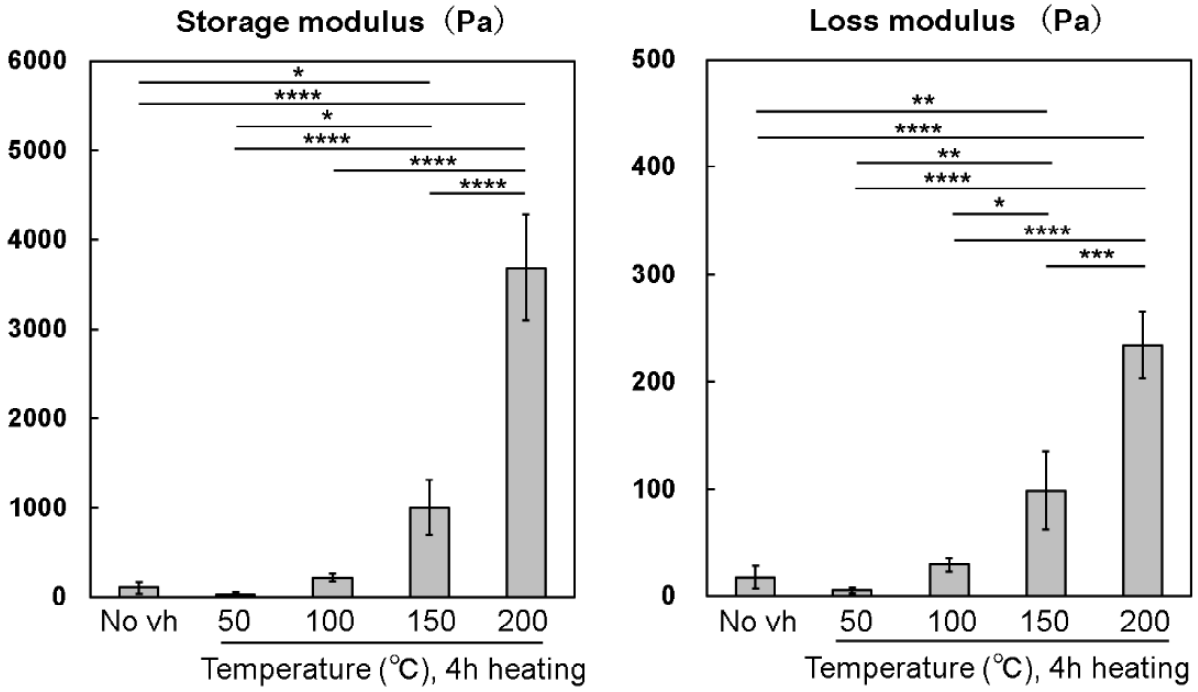

Fig. 4 Rheological measurements for samples treated with or without vacuum heating (dehydrothermal treatment) for 4 h at the different heating temperatures. Mean with standard deviation (n=3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$（ANOVA with Tukey test）.

temperature change during dehydrothermal treatment using vacuum heating for Ec-GS under the same 4 h treatment (Fig. 4). Due to technical difficulties, only the Ec-GSs were elucidated in this study. Both the storage and loss moduli increased remarkably beyond 100°C, whereas negligible changes were observed at 100°C or less (Fig. 4). In the ATR-FTIR measurements, subtle changes were observed at 1357 cm^{-1} in the spectra of the Ec-GS treated at 200°C for 4 h in comparison with that of intact Ec-GS without vacuum heating (Fig. 2). No other drastic changes were observed in the ATR-FTIR spectra before and after vacuum heat treatment when the temperature was increased to 200°C.

3. Effect of time during vacuum heating treatment

Subsequently, we evaluated the effect of heating time from 4 to 24 h under vacuum heating at the same temperature of 150°C. SEM images of the representative EGCG-conjugated gelatin parts in Ec-GS and Ec-GS-β are shown in Fig. 5. As with the Ec-GSs without vacuum heating treatment (No vh in Fig. 3), plate-like and fibrous structures were observed (Figs. 3 and 5); expanding the time up to 24 h did not induce significant changes in the microstructures (Fig. 5A). In the high magnification images (×3,000), there seems to be no remarkable change in the surface topography of the EGCG-conjugated gelatin parts in all samples, which retained a smooth surface without the addition of macropores or roughness. The storage and loss moduli gradually increased after 8 h and reached their highest values at 16 h, after which they decreased at 24 h (Fig. 6). However, the ATR-FTIR spectra showed no significant changes in the peaks, even after the longest 24 h of vacuum heating treatment (Fig. 2).

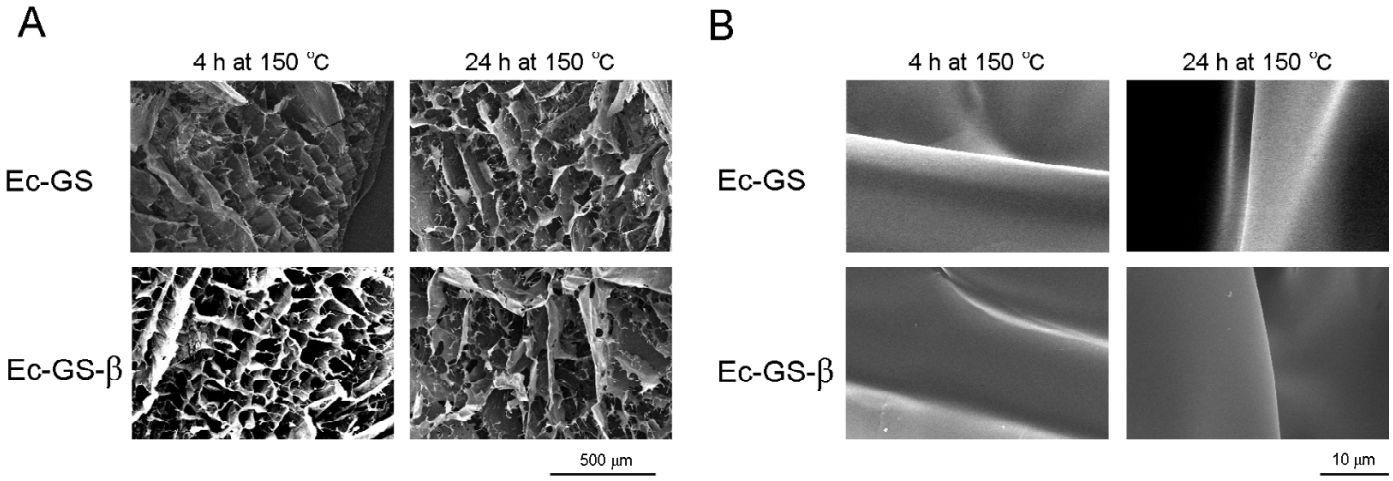

Fig. 5 Representative scanning electron microscopic images of samples treated with vacuum heating (dehydrothermal treatment) at 150°C for different heating times. Ec-GS: epigallocatechin-conjugated gelatin sponges; Ec-GS-β: Ec-GS with β-TCP granules.

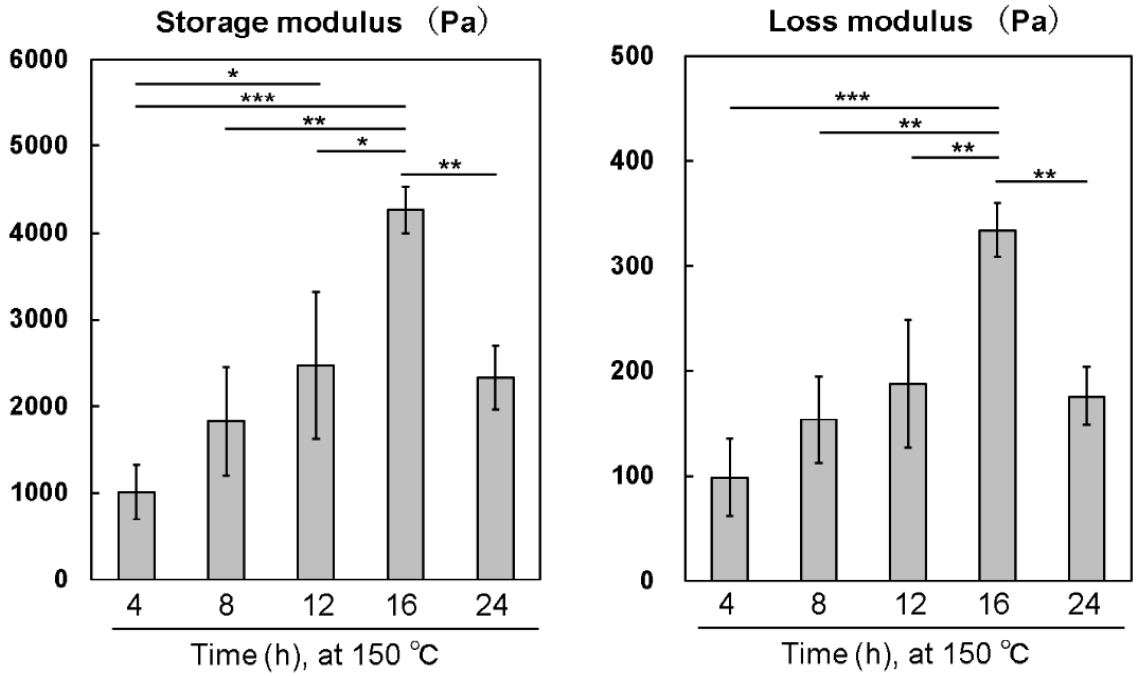

Fig. 6 Rheological measurements for samples treated with vacuum heating treatment (dehydrothermal treatment) at 150°C under different heating times. Mean with standard deviation (n=3). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$（ANOVA with Tukey test）.

Discussion

The present study reveals that increasing the temperature beyond 100°C or extending the heating time up to 16 h at 150°C under vacuum heating increased the viscoelasticity of Ec-GSs. In contrast, there were no significant changes in the surface morphology, showing plate-like or fibrous shapes at the EGCG-conjugated gelatin parts in the sponges or their surface topography.

Intrinsic Ec-GSs and Ec-GS-βs (without vacuum heating) prepared in this study using the aqueous chemical synthesis methods showed plate-like and fibrous structures with porous structures similar to the study reported previously (Fig. 3) [17]. There is consensus that surface roughness, pore size, porosity, and the interconnection of pores markedly affect bone regeneration [19-21]. The gas adsorption method is a conventional method for estimating the pore size or porosity of materials [23], whereas this method is commonly unsuitable to use for soft materials deforming easily, such as Ec-GS and Ec-GS-β. Consequently, we refrained from obtaining detailed quantitative information about the changes in the pores. However, in the SEM observations, we could not find drastic changes in the microstructure or surface topography of either material when the heating temperature was increased or the time was extended. There were no remarkable changes in the plate-like and fibrous structures at low magnification or the smooth surfaces at high magnification, such as the appearance of the pore structure on the surface (Figs. 3 and 5). These results are similar to those reported in other study, which exhibited poor changes in surface structure after vacuum heat treatment at 160°C for 48 h to gelatin/genipin [24]. Ec-GSs contained a limited EGCG (0.28 w/w%) relative to gelatin. Altogether, although we could not elucidate the change in pore size, these results indicate that the increase in temperature and heating time to a certain level in the vacuum heating treatment scarcely affects the surface topography of EGCG-conjugated gelatin at least when it contains a small quantity of EGCG.

Rheological measurements using Ec-GSs demonstrated that the storage and loss moduli hardly changed at heating temperatures of 100°C or less; the shape of the measured samples dissolved without forming a hydrogel (Figs. 4 and 6). In contrast, the storage and loss moduli increased with increasing heating temperature and heating time at 150°C; the shape of the measured samples remained hydrogel. This result is considered the same as that for chemically modified gelatin, in which the storage and loss moduli increase as the number of cross-linking reaction points increases [25]. These results suggest that, at the very least, conditions higher than 100°C are necessary to induce thermal cross-linking between gelatins. A crosslinked structure seems to have been gradually formed in the Ec-GSs by increasing the temperature above a certain level and extending the reaction time up to 16 h.

When the heating time was extended beyond 16 h at 150°C, both the storage and loss modulus values decreased (Fig. 6). Although there are few reports on this phenomenon, a previous study reported that heating causes gelatin decomposition to some extent [26]. Although further investigation is inevitable, the long dehydration heat treatment beyond 16 h possibly resulted in the decrease of storage and loss moduli relatively due to the cleavage of the main chain on the gelatin by the dominant progression of gelatin degradation over the cross-linking reaction.

Our results showed that the viscoelasticity of gelatin conjugated with EGCG could be improved by adjusting the heating temperature and heating time of vacuum heat treatment. Further clarification is essential for deepening the understanding of these findings. For example, the samples used in this study contained only small amounts of EGCG. Increasing EGCG may yield different outcomes. Both EGCG and gelatin-containing hydroxyl groups have the potential to be involved in thermal cross-linking. It is still unclear which hydroxyl group dominantly contributes to the thermal cross-linking in Ec-GS, which can affect the change in the viscoelastic properties. Additionally, as mentioned above, gelatin from different animal origins exhibits different physical properties [3]; different optimal temperatures and times for inducing the cross-linking will exist for these gelatins. However, our findings would provide insights and guidance for designing new EGCG-or polyphenol-conjugated gelatin materials.

Conclusion

The microstructures, surface topographies, and mechanical properties of biomaterials directly affect their outcomes when they are used in tissue engineering or disease treatment. In this study, we evaluated the effect of the heating temperature and time at vacuum heat treatment (dehydrothermal treatment) for EGCG- conjugated gelatin sponges with or without β-TCP granules (Ec-GS and Ec-GS-β). Negligible alterations in microstructure or surface topography were observed under the conditions of temperature elevation to 200°C for 4 h or time extension to 24 h at 150°C. However, when Ec-GSs were subjected to the dynamic viscoelasticity test, the storage and loss moduli of Ec-GS significantly changed beyond 100°C. Expanding the heating time up to 16 h at 150°C increased those moduli, whereas it decreased at 24 h. The increase in these moduli raises the possibility that physical cross-linking is partially formed in the Ec-GSs, whereas the decrease could be due to gelatin degradation. Different degrees of cross-linking seems to be a usable technique for fabricating materials with distinct solubilities, which can be applied to prepare tailor-made materials against distinct tissues. These data will contribute to the development of new gelatin-based or other biopolymer (such as collagen) materials using dehydrothermal treatment and vacuum heating techniques.

Acknowledgments

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Conflict of interest

The study received financial support from Toagosei Co. Ltd., and it is important to note that there are no conflicts of interest associated with this funding.

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Corresponding author:
Prof. Yoshitomo honda, D.D.S., Ph.D
Department of Oral Anatomy,
Osaka Dental University
8-1, Kuzuha hanazono cho, Hirakata,
Osaka 573-1121, Japan.
Tel: +81-72-864-3013
E-mail: honda-y@cc.osaka-dent.ac.jp