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Bioactive microspheres produced from gelatin–siloxane hybrids for bone regeneration

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Abstract In this study, we produced a novel microsphere with a hybridized composition of gelatin and siloxane which is bioactive and degradable for the applications in bone regeneration fields. A solution of gelatin organic and siloxane inorganic containing calcium chloride was formulated into microspheres in an oil bath mediated by a surfactant. Following the processes of hydration, gelation and solidification, well-shaped spherical particles were produced with sizes of a few to hundreds of micrometers (68 μm on average). The obtained microspheres were highly stable in an aqueous solution due to the in situ cross-linking of the siloxane with gelatin chains, which eliminated the additional cross-linking step generally used in pure gelatin. The hybridized microspheres exhibited rapid induction of apatite-like crystals on their surface with incubation in a simulated body fluid, suggesting an excellent bone bioactivity in vitro. It is considered that the newly developed organic–inorganic microspheres may be useful for the regeneration of skeletal defects.

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1 Introduction

Microspherical particles have been widely used as a delivery vector of drugs, proteins and cells for the regeneration and repair of damaged and defective tissues [1–7]. The microspherical form, compared to other formulations, is easily tuned to the size and shape of defects and thus has been widely used in direct-filling defective sites and as injectable materials. For the last years, many degradable polymers in natural or synthetic base have been formulated into microspheres. In vitro and in vivo studies have shown some promise of using those polymeric microspheres in the treatment of skeletal defects. However, the polymeric composition is not considered to be the optimal material choice for the bone cell growth and osteogenic stimulation.

On the other hand, bioactive inorganics such as calcium phosphates and bioactive glasses have shown better performance in the bone cell responses and bone matrix production [8–10]. The activity to bone formation by those materials, namely 'bioactivity', is basically understood as the ability to form a bone-like mineral phase at the material surface in direct contact with body fluid, which provides favorable substrate conditions for bone cell development and bone matrix synthesis [8]. Above all, the composition of materials is of particular importance for the bioactivity.

Up to date, those bioactive compositions have been developed in the forms of powders, irregular-shaped granules and small-sized porous blocks, for use as bone defect fillers and tissue engineering matrices [9–11]. However, the ceramic or glass compositions mainly have the processing limitations to be developed into a product with well-controlled size and shape due to the brittleness and limited shape flexibility or moldability. As a result, the

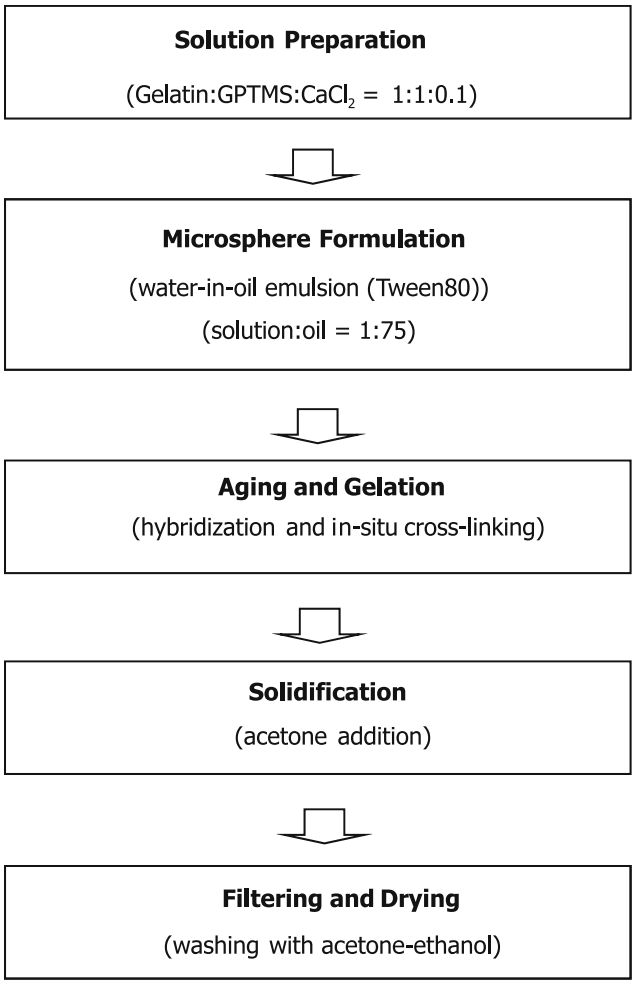

Fig. 1 Schematically shown the experimental procedures of producing gelatin–siloxane hybridized microspheres

microspherical formulation with those bioactive compositions has rarely been studied. Compared to the irregular-shaped granules, the microspheres with controlled size and shape are considered to be more useful as implantable or injectable bone regenerative fillers, or as delivery vectors for drugs or cells [3].

In this study the authors produced microspherical particles of which the composition is bioactive and degradable. For this, the hybridized sols of gelatin organic and siloxane inorganic derived from alkoxysilane-based polymer were used as the precursors of the microspheres. Previous studies on the siloxane-based organic hybrids such as siloxane–gelatin and siloxane–chitosan have proved their ability to form bone-mineral like apatite on the surface under simulated biological conditions [12–15]. Herein, we produced the gelatin–siloxane composition into microspheres for the applications in the bone regeneration field. The processing tools to produce the gelatin–siloxane hybridized microspheres and their characteristics are described.

2 Materials and methods

As the reagents for the hybrid solution, gelatin (type B, from bovine, Sigma), 3-(glycidoxypropyl) trimethoxysilane (GPTMS) (98%, Aldrich), calcium chloride ($CaCl_2$) (Anhydrous, 99.99%, Aldrich), and HCl (Aldrich) were used. Gelatin was first dissolved in 0.1 N HCl–water solvent at 12.5 wt.%, and then GPTMS was added to the solution and followed by a vigorous stirring. The ratio of gelatin to GPTMS was fixed at 1. In particular, calcium chloride was added at 5 wt.% with respect to the gelatin–siloxane as a calcium source to improve the bioactivity of the hybrids, which was based on previous work using calcium nitrate [12].

The mixture solution homogenized for 2 h was used for the microsphere generation. Five milliliter of the solution was added dropwise into an oil bath (olive oil, Aldrich)

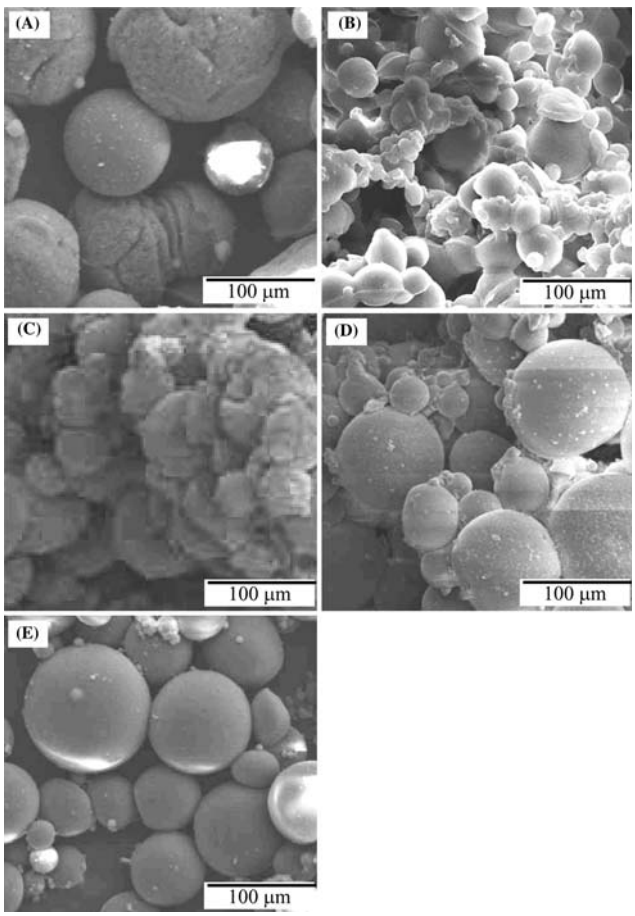

Fig. 2 SEM morphologies of the produced microspheres at different conditions: gelatin–siloxane obtained (**a**) without calcium chloride, and (**b–e**) with calcium chloride. Addition of calcium chloride hampered the separation of particles significantly (**b**). However, the mediation of Tween-80 surfactant improved the generation of individual particles with well-developed spherical form (**c–e**): Oil bath contained Tween-80 at (**c**) 0.5%, 0.75% and (**e**) 1%

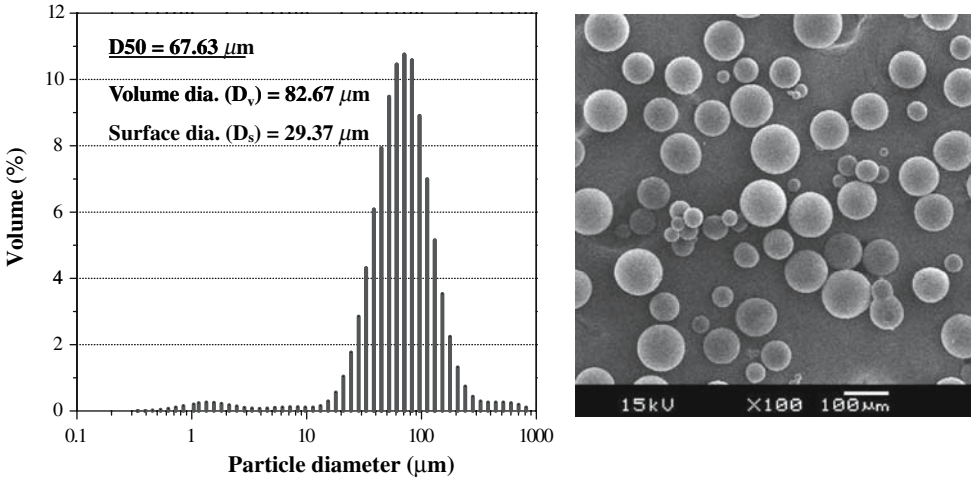

Fig. 3 Particle size distribution of the gelatin–siloxane hybridized microspheres, showing an average diameter of 67.63 μm. SEM image of the sample is also shown

within a 500 mL flask containing a surfactant Tween-80 (Aldrich) while stirring at 500 rpm. The ratio of solution to oil was set at 1:75, and the concentrations of the surfactant were varied to observe the effect of the surfactant. The microsphere-formed bath was aged for up to 36 h to improve the gelation of the hybrid microspheres. After aging, cooled acetone was added into the bath to extract remnant water and harden the microspheres. The solidified microspheres were filtered fully with acetone–ethanol and vacuum-dried for further uses.

The experimental procedures for the production of the bioactive hybrid microspheres are schematically shown in Fig. 1.

The morphology of the obtained microspheres was examined by means of scanning electron microscopy (SEM; JSM6330F, JEOL). The size distribution of the microspheres was observed with particle size analyzer (Mastersizer MSX64, Malvern Instrument). The chemical bonding structure of the microspheres was investigated with Fourier transformed infrared spectroscopy (FT-IR; Perkin-Elmer). To confirm the stability, the microspheres were soaked in distilled water at 37 °C, and then observed with SEM. The in vitro bioactivity of the microspheres was examined with incubation of the samples in body simulating medium (1.5SBF), and then the surface was analyzed to observe the induction of apatite-like mineral phase.

3 Results and discussion

The SEM morphologies of the hybrid microspheres obtained at different conditions are shown in Fig. 2. It was

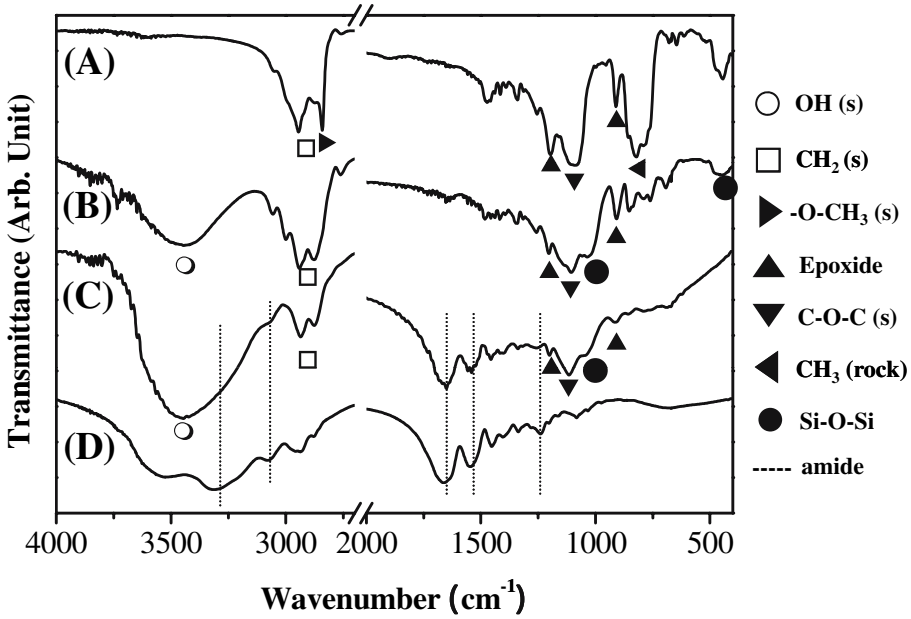

Fig. 4 FT-IR spectra of (a) pure GPTMS without gelation, (b) pure GPTMS with gelation, (c) gelatin-GPTMS with gelation, and (d) gelatin–GPTMS without gelation. Gelation was performed at 40 °C for 36 h in case of gelatin–GPTMS microsphere and for 12 days in case of pure GPTMS. The Si–O–Si siloxane bands in the gelatin–GPTMS were similarly observed as those in the pure GPTMS due to gelation

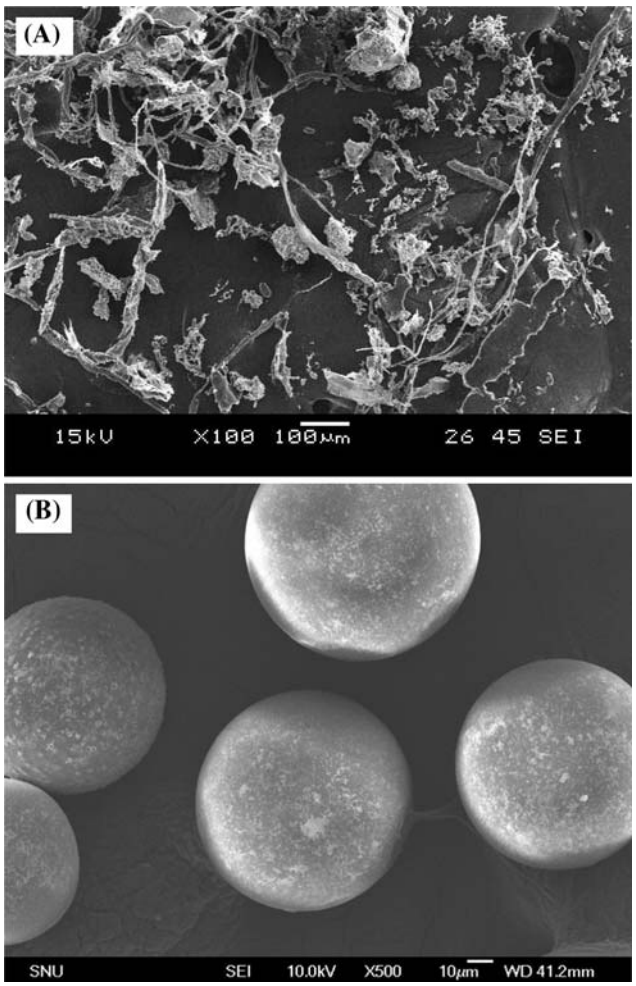

Fig. 5 The gelatin–siloxane microspheres poorly aged for 1 h (**a**) were completely disintegrated within a day in distilled water, however, when aged fully for 36 h (**b**), the microspheres maintained their initial morphology well, when incubated in distilled water for 4 weeks

observed that pure gelatin without the addition of GPTMS was easily produced into microspheres in an oil bath. Moreover, the GPTMS-added gelatin hybrid composition (gelatin:GPTMS = 1:1) also could be generated into microspherical particles with individual particles being separated well (Fig. 2a). However, the addition of calcium chloride made the microparticles severely agglomerated, resulting in the spherical shape not being maintained (Fig. 2b). In practice, calcium chloride is required to improve the bioactivity of the siloxane–gelatin system, as has been previously suggested [12, 13]. However, the added calcium chloride was considered to be deleterious to the stability of microsphere/oil interface because of the ionic effect, so the spherical shaping could not be attained with the hybrid composition.

To overcome this problem, the surfactant Tween-80 was added into the hybrid solution. The addition of small amounts of Tween-80 was observed to significantly improve the microspherical formation of the hybrid composition (Fig. 2c–e). The minimal concentration of the surfactant was observed to be $\sim$1 wt.%, at which the well-shaped spherical particles could be produced without being agglomerated.

The size distribution of the hybrid microspheres of gelatin–siloxane hybrid containing calcium chloride obtained at fixed experimental conditions is shown in Fig. 3, including the image of test sample. Spheres with sizes of a few to hundreds of micrometers were produced, and the average size of the microspheres was measured to be $\sim$68 μm. In practice, many experimental variables, such as sol concentration and composition, water to oil ratio, surfactant concentration, and rotating speed, determine the size of the particles. It is generally accepted that the size of the particles increases as the ratio of water to oil increases, the surfactant concentration decreases, and the rotating speed decreases [4].

One important thing to consider is the water stability of the microspheres. As the gelatin normally degrades rapidly in water, it requires additional cross-linking process for the biomedical applications. In practice, we observed that the pure gelatin microspheres as-produced without the subsequent cross-linking process rapidly disintegrated in water (within an hour). However, the addition of GPTMS into gelatin was observed to significantly improve the stability of the microspheres. This was due to the in situ cross-linking of gelatin associated with the siloxane groups. During the gelation step in the fabrication of gelatin–siloxane microspheres, the silane groups in GPTMS hydrolyze and condense to form siloxane groups, which further link gelatin chains to form a hybridized network [12–15].

Figure 4 shows the FT-IR spectra of the gelatin–siloxane hybrid microspheres underwent gelation process at 40 °C. Data on pure GPTMS (the siloxane precursor) were also presented for comparison purpose. In the pure GPTMS, an additional siloxane (Si–O–Si) band at $\sim$1030 cm^{-1} appeared after the gelation (compare Fig. 4a and b), which resulted from the series of reactions of hydration and condensation of the GPTMS trialkoxysilane groups within a water-based acidic solution. When the gelatin was added into the GPTMS without the subsequent gelation step (Fig. 4d), only amide bands associated with gelatin occurred in conjunction with GPTMS-related bands. However, in the gelatin–GPTMS underwent gelation at 40 °C for 36 h, the siloxane band developed similarly as in the case of pure GPTMS underwent gelation. Moreover, the amide bands associated with gelatin were shown to be attenuated in the gelation-performed sample. Those illustrate the gelation step is required to create siloxane groups in the GPTMS [12], and furthermore during the gelation the amide chains bridge with siloxane groups to form a hybridized network [14]. In practice, it has been studied that the epoxy groups in

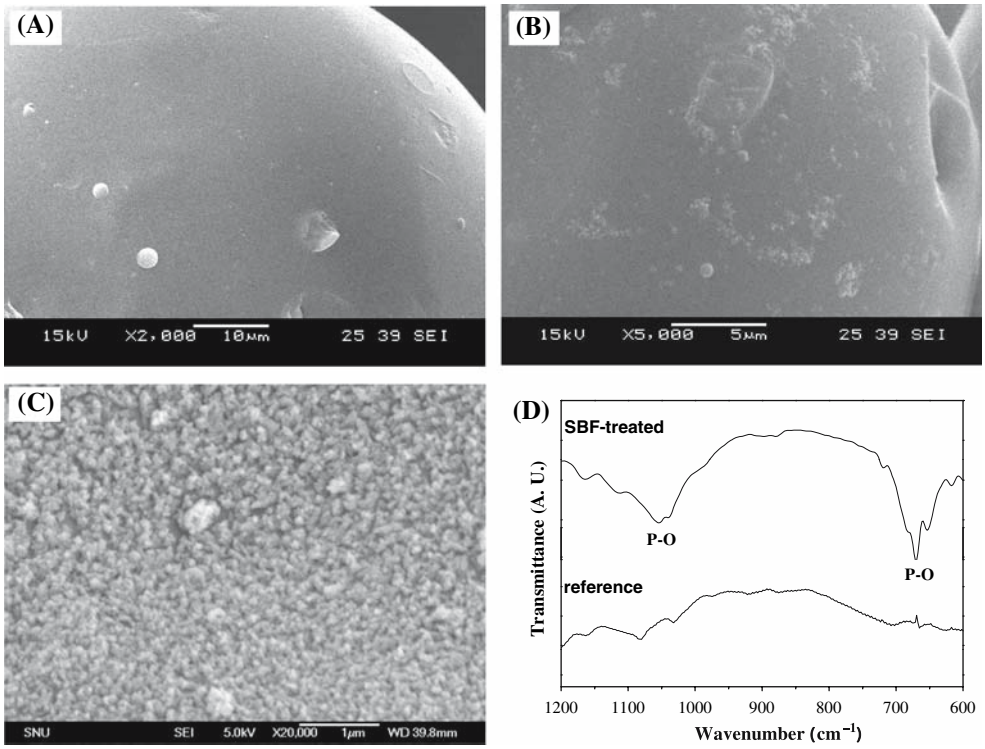

Fig. 6 In vitro bone bioactivity test of the gelatin–siloxane microspheres following the incubation in a simulated body fluid: SEM image of the microspheres (**a**) without incubation, (**b**) incubated for 12 h, and (**c**) for 72 h, which showing the significant precipitation of apatite-like minerals, and (**d**) FT-IR spectrum of the minerals in the SBF-treated sample, showing the evolution of P–O bands (SBF-treated), while no P–O band being observed in the as-prepared microsphere (reference)

GPTMS react with the amino groups of natural polymers including gelatin through the acid-catalyzed amino-oxirane addition reaction, while the trialkoxysilane groups hydrolyze to form silanol groups which were further condensed to form siloxane networks [12–15]. Moreover, other alkoxysilane-based polymers except GPTMS have been used to introduce covalent bonding between the organic and inorganic interfaces [16, 17].

The water stability of the gelatin–siloxane microspheres was observed after immersion of the sample in distilled water, as shown in Fig. 5. When the microspheres were poorly aged (for 1 h of gelation) they were completely disintegrated in the distilled water solution within a day (Fig. 5a). However, after the gelation for 36 h, the microspheres maintained their as-produced morphology with incubation in distilled water over 4 weeks (Fig. 5b), illustrating the high stability in water, and their possible uses in biomedical applications. To obtain the hybrid microspheres with high stability in water, the gelation process was observed to be last at least for 36 h, which was based on the change in the siloxane group from the FT-IR spectra (data not shown).

The bioactivity of the hybrid microspheres was briefly examined after immersion of the sample in a simulated body fluid (1.5SBF). Figure 6a–c shows the morphology change of the microspheres during incubation in 1.5SBF. After incubation for 12 h, only a slight change was observed on the surface of microspheres (Fig. 6b). A prolonged incubation (for 72 h) significantly changed the surface morphology (Fig. 6c), showing a number of nanocrystalline precipitates covered the microsphere surface. The FT-IR spectrum (Fig. 6d) of the precipitated surface of the SBF-treated sample shows the evolution of P–O bands, which being in direct contrast to that of the as-prepared microsphere (reference), and this is deemed to the apatite-like crystallines formed on the surface. In practice, the gelatin–siloxane composition, when made into porous foam, has previously been reported to induce apatite-like minerals on the surface [13]. In this study, we again confirmed the hybrid composition retained in vitro bone bioactivity, finding its usefulness in the bone regeneration fields. In particular, the microspherical form of the gelatin–siloxane hybrid is considered to find direct uses in filling defective skeletal tissues as an implantable or injectable type.

4 Conclusions

A novel microsphere with a bioactive hybrid composition of gelatin and siloxane was produced. The water-based

hybrid sol of gelatin and siloxane containing calcium was formulated into spherical particles within a surfactant-containing oil bath. The obtained particles showed a well-developed spherical shape and had an average diameter of 68 μm under controlled conditions. The hybrid microspheres maintained water stability due to the in situ cross-linking of gelatin with siloxane. In vitro bone bioactivity test in a simulated body fluid showed the hybrid microspheres rapidly induced apatite-like minerals on their surface. The bioactive hybrid microspheres may be applicable as implantable or injectable materials in the regeneration of defective skeletal tissues.

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