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Effects of surface carboxylic acid groups of cerasomes, morphologically stable vesicles having a silica surface, on biomimetic deposition of hydroxyapatite in body fluid conditions

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Abstract Biomimetic mineralization of supramolecular scaffolds consisting of biomolecules or their analogues has received much attention recently from the viewpoint of creation of novel biomaterials. This study investigated biomimetic deposition of hydroxyapatite (HAp) on cerasomes, morphologically stable organic–inorganic hybrid vesicles. Scanning electron microscopy, energy-dispersive X-ray spectroscopy, and X-ray diffraction studies revealed that the pristine cerasomes induced heterogeneous nucleation of HAp when they were immersed in 1.5SBF, a solution having 1.5 times higher ion concentration than that of a simulated body fluid (SBF). The HAp deposition was further accelerated when dicarboxylic and monocarboxylic acid groups were displayed on cerasome surfaces. These carboxylic acid groups are expected to enhance calcium ion binding to the cerasome surface, causing an increase of HAp nucleation sites. At lower surface concentrations on the cerasome surface, the dicarboxylic acid group is apparently more effective for HAp deposition than the monocarboxylic acid group. The resultant HAp-cerasome hybrids are useful as biocompatible materials having unique properties deriving from the lipid bilayer structure of the cerasomes.

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1 Introduction

Organic–inorganic nanohybrids are attractive materials because of their structural and functional characteristics arising from the combination of the organic and inorganic components. The use of molecular self-assembly for construction of organic–inorganic nanohybrids enables precise control the unit structure of the hybrids [1, 2]. The resulting nanohybrids are expected to exhibit good biocompatibility if the organic components are biomolecules or their analogues and/or the inorganic components are bioceramics.

Lipid bilayer vesicles, which are well known as a biomembrane model, consist of a supramolecular structure of amphiphilic lipid assemblies [3, 4]. They have aqueous interior phases. In addition, their bilayer parts have structural flexibility that enables a gel-to-liquid-crystalline phase transition while maintaining their vesicular morphology. For use as biomembrane models and as a biomaterial in applications such as carriers for drug delivery systems, they have been investigated extensively. Researches into creation of organic–inorganic nanohybrids using

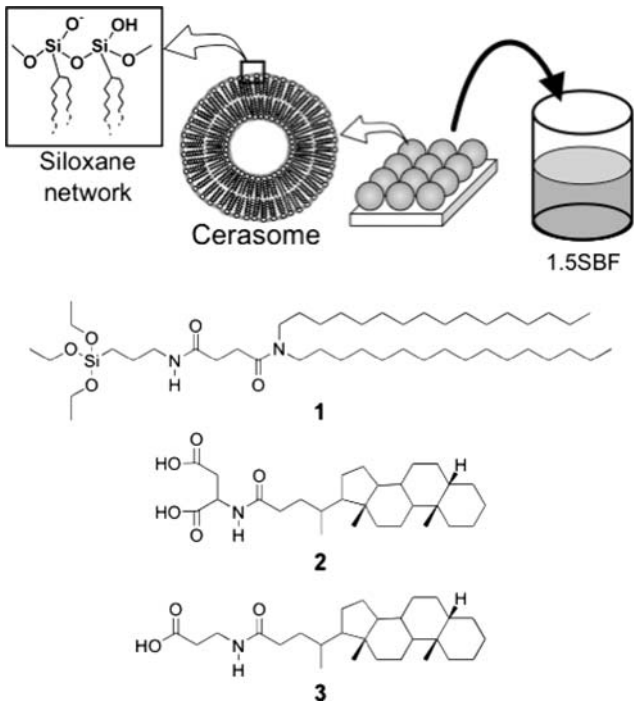

Fig. 1 Schematic illustration of monolayer assembly of cerasomes on a solid substrate and chemical structure of the lipids employed in this study

lipid vesicles have also intensified recently. Several groups have reported the coating of vesicular surfaces with ultrathin inorganic layers of silica [5, 6] or calcium phosphate [7]. They carefully chose conditions for sol–gel condensation or crystallization on the vesicular surface; otherwise, the formation of inorganic layer disturbs the vesicular structure because of its structural rigidity.

Using a different approach, we recently developed an organic–inorganic vesicular nanohybrid, the cerasome, from lipid-like molecules such as **1** (Fig. 1) [8, 9]. Cerasomes show remarkably high morphological stability because of their atomic layer of surface siloxane networks. Cerasomes maintain their vesicular structure in the presence of membrane-solubilizing surfactants [9–11]. Moreover, they can form a three-dimensional multicellular architecture on solid substrates [12]. Neither of these capabilities is available when using conventional lipid vesicles. Further modification of cerasomes with other nanocomponents results in the construction of hybrid vesicles having specific functions. From this viewpoint, we created an additional ultrathin inorganic layer on the cerasome surface using alkoxide compounds such as tetraethoxysilane (TEOS) [13] and titanium tetrabutoxide [14]. The titania-coated cerasomes obtained using the latter system exhibit photocatalytic activity. Very recently, using electroless plating technique, we created cerasomes having ultrathin metallic surface layers [15, 16]. These results ensure that other surface coating techniques will be available to cerasomes because of their high morphological stability.

To create organic–inorganic hybrid biomaterials, the processes inspired by biomineralization [17] are attractive and extremely useful because they proceed under mild conditions. Furthermore, the design of the organic scaffold surfaces can control the deposition behavior of inorganic components [18, 19]. Hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$) is the major inorganic component of bones. It has been investigated widely for use as a biomaterial and other practical applications such as absorbent filters [20]. To evaluate the activity of HAp mineralization on scaffold materials under physiological conditions, Kokubo et al. developed a simulated body fluid (SBF) with similar inorganic ion concentrations to those of human plasma. It is supersaturated for HAp [21, 22]. Using SBF and 1.5SBF, which has all ion concentrations 1.5-times higher than those of SBF, various scaffolds were evaluated for their HAp deposition activity [23–32]. Results of these studies revealed that displaying functionalities such as silanol [23–25] and carboxylic acid [25–31] groups—which can bind Ca^{2+}, a HAp component, onto the scaffold surfaces—is effective to enhance HAp deposition. Alternatively, these SBF systems have been used as a technique to deposit biomimetic HAp on various materials' surfaces. However, to our knowledge, the literature includes no report of soft colloidal capsules with organic–inorganic nanostructures used as a scaffold—either for deposition of HAp or other bio-related minerals—using such a mineralization process.

To create novel biocompatible hybrid materials, biomimetic HAp deposition on cerasome surfaces in 1.5SBF was investigated. Furthermore, the effect of displaying carboxylic acid groups on cerasome surfaces on HAp deposition was evaluated, especially with respect to their surface density. The advantages of using cerasomes when creating such materials are also discussed.

2 Materials and methods

2.1 Chemicals

Unless otherwise stated, all reagents and chemicals were obtained commercially and used without further purification: 3-aminopropyltriethoxysilane (APS) from Shin-Etsu Chemical Co. Ltd.; 5β-cholaic acid from Sigma Chemical Co.; *N*,*N*-diisopropylethylamine, from Aldrich Chemical Co. Inc.; 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), from Dojindo Laboratories. A nonionic surfactant Triton-X 100 (TX-100) and chemicals used for the preparation of 1.5SBF were purchased from Nacalai Tesque, Inc. Other chemicals were obtained from Wako

Pure Chemical Industries Ltd. The 1.5SBF (Na^+ 213.0, K^+ 7.5, Mg^{2+} 2.3, Ca^{2+} 3.8, Cl^- 221.7, HCO_3^- 6.3, HPO_4^{2-} 1.5, and SO_4^{2-} 0.8 mM, pH 7.25) was prepared using Kokubo's method [21, 22]. The cerasome-forming lipid, *N*-[*N*-(3-triethoxysilyl)propylsuccinamoyl]dihexadecylamine (**1**) was synthesized in our laboratory as described in previous reports [8, 9]. Syntheses of cholic acid derivatives having carboxylic acid groups (**2** and **3**) (Fig. 1) are described in *Supplementary Materials*. Solvents used for synthesis were purified through distillation. Water used for the experiments was distilled (Autostill WS33; Yamato Scientific Co. Ltd.) and deionized (Milli-Q Labo; Nihon Millipore Ltd.).

2.2 Preparation of cerasomes and their assembly on substrates

Cerasomes derived from **1** (cerasome **1**), and from mixed lipids of **1** and **2** (cerasome (**1–2**)) or **1** and **3** (cerasome (**1–3**)) were prepared using the ethanol sol injection method [13]. An ethanol sol of the lipid(s) (**1** or (**1** and **2**) or (**1** and **3**)) with the ratio of lipid:ethanol:H_2O:HCl = 1:200:19:0.03 was stirred for 12 h at room temperature using a vortex mixer. It was then injected into HEPES buffer (pH 7.25) to produce a final lipid concentration 0.3–0.5 mM. The obtained cerasomes were evaluated using the following physical measurements. Hydrodynamic diameters (D_{hy}) of cerasomes were measured using dynamic light scattering (DLS, DLS-6000HL; Otsuka Electronics Co. Ltd.). Scanning electron microscopy (SEM, JSM-6301F; JEOL) used an acceleration voltage of 15 kV. The SEM samples were also analyzed using energy-dispersive X-ray analysis (EDX, JED-2201F; JEOL). For EDX analyses, SEM images were obtained without metal sputtering of specimens. The cerasome zeta-potentials were obtained (ELS-6000; Otsuka Electronics Co. Ltd.) in aqueous NaCl (10 mM). The morphological stability of cerasomes was evaluated according to the light scattering intensity for the vesicular solution at 400 nm upon the addition of TX-100 [9–11]. Unless otherwise stated, the cerasome dispersions were incubated for about 24 h at room temperature to develop surface siloxane networks of the cerasomes prior to their use for the following experiments.

Monolayer assembly of the cerasomes on solid substrates was performed in a similar manner to that used for layer-by-layer assembly of cerasomes [12]. Potassium poly(vinyl sulfate) (PVS) and poly(diallyldimethylammonium chloride) (PDDA) were assembled alternately on an APS-coupled glass substrate to form a precursor film whose outermost surface was PDDA. This substrate was then immersed in a cerasome dispersion to form a monolayer assembly of cerasomes on its surface. The substrate was finally rinsed using milli-Q water and dried by nitrogen flushing.

2.3 Deposition of HAp on cerasome surfaces

A cerasome-assembled substrate was immersed into 1.5SBF at 36.5°C. After an appropriate immersion time, the substrate was washed well with milli-Q water and dried by nitrogen flushing. The surface of the resultant substrate was observed using SEM and analyzed using EDX. The substrate was also evaluated using XRD analyses (RINT-2200VL; Rigaku Corp.).

In a separate experiment, 1 ml of dispersion containing cerasome **1** or cerasome (**1–2**) was added to 2 ml of 1.5SBF to produce the same final concentration of inorganic components as those of SBF (Na^+ 142.0, K^+ 5.0, Mg^{2+} 1.5, Ca^{2+} 2.5, Cl^- 147.8, HCO_3^- 4.2, HPO_4^{2-} 1.0, and SO_4^{2-} 0.5 mM). During this process, the change of D_{hy} of the cerasome in the dispersion was monitored using DLS. As a reference, the change of D_{hy} of the cerasome in 2.5 mM calcium chloride solution was also monitored.

3 Results and discussion

3.1 Preparation and characterization of cerasomes

Cerasomes are anticipated for application as novel scaffolds for HAp deposition because they have silica-like surfaces and high morphological stability. In addition to evaluation of the HAp deposition ability of cerasomes, we investigated the effects of introducing carboxylic acid groups on cerasome surfaces. Two main reasons exist for introducing the (di)carboxylic acid unit into the cerasomes. One is that bone Gla proteins, non-collagen type bone proteins, have γ-carboxyglutamic acid (Gla), which is known as an important factor for bone formation and which is known to display a dicarboxylic acid group [33]. Another reason is that results of previous studies suggest that the carboxylic acid group is effective for HAp deposition [25–31]. In both cases, calcium ion binding to the carboxylate moieties formed nucleation cites for HAp. In this study, to display carboxylic acid groups on the cerasome surface, we used cholic acid derivatives having a dicarboxylic and monocarboxylic acid headgroup, **2** and **3**. We also prepared "mixed" cerasomes from these lipids using cerasome-forming lipid **1**.

The hydrodynamic diameter (D_{hy}) and polydispersity index (*p.i.*) of cerasomes derived from the cerasome-forming lipid **1** (cerasome **1**), the mixture of **1** and **2** (cerasome (**1–2**)), and that of **1** and **3** (cerasome (**1–3**)), in HEPES buffer (pH 7.25) are presented in Table 1. The D_{hy} of cerasome **1** was 280 nm and those of cerasome (**1–2**) and cerasome (**1–3**) were, respectively, 260 nm and 300 nm. Such a slight difference in D_{hy}s among these cerasomes might arise from the difference in critical packing parameters [34] between **2** and **3**.

Table 1 Hydrodynamic diameters (D_{hy}s) of cerasomes[a]

Sample	D_{hy}/nm	*p.i.*[b]
Cerasome **1**	280	0.29
Cerasome (**1**–**2**)[c]	260	0.16
Cerasome (**1**–**2**)[c,d]	240	0.22
Cerasome (**1**–**3**)[c]	300	0.30

[a] In HEPES buffer (pH 7.25)

[b] Polydispersity index

[c] 10 mol% of **2** or **3** in total lipids

[d] After 6 h incubation in the presence of TX-100

The obtained cerasomes showed morphological stability against a nonionic surfactant triton TX-100 after an appropriate incubation time to develop surface siloxane network. Cerasome **1** in HEPES buffer (pH 7.25) needed 6 h to obtain sufficient morphological stability (data not shown). This is comparable to our previously obtained results showing that it took 10 h to make cerasomes morphologically stable at pH 7 [11]. For mixed cerasomes, incorporation of lower amounts of **2** or **3** (up to 50 mol% of total lipids) did not strongly affect morphological stability of cerasomes against TX-100 (one example is presented in Table 1). Much higher ratios of **2** or **3** decreased the morphological stability of cerasomes (data not shown). This is reasonable because higher amounts of **2** or **3**, i.e. the lower amount of **1** in the mixed cerasomes, caused a decrease in the development of siloxane networks on vesicular surfaces.

3.2 Deposition of HAp on cerasome surfaces under body fluid conditions

To investigate the HAp deposition on the cerasome surface, we used cerasome-assembled solid substrates because of their relative ease of characterization compared to the corresponding colloidal dispersions in the present mineralization system. The use of vesicular assemblies on substrates also underscores one advantage of cerasomes: their enhanced morphological stability [9–11]. Furthermore, the resultant HAp/cerasome superstructures themselves are interesting from the viewpoint of hybrid biomaterials. We assembled the cerasomes as a monolayer on the substrates in a manner similar to that for the case of their multilayer assembly [12].

After one-week immersion of the cerasome **1**-assembled substrate into 1.5SBF at 36.5°C, deposition of inorganic components on the substrate was visible to the naked eye (Fig. 2a). The SEM images of the substrate show that disk-like crystals were deposited all over the substrate surface (Fig. 2b). Surface analyses using EDX revealed that the crystals contained calcium and phosphate. The average value of the Ca/P ratio for this sample was 1.8, which is slightly higher than that for HAp (1.67), which is comparable to previous studies using SBF or 1.5SBF. The reason for the higher Ca/P value is probably the formation of

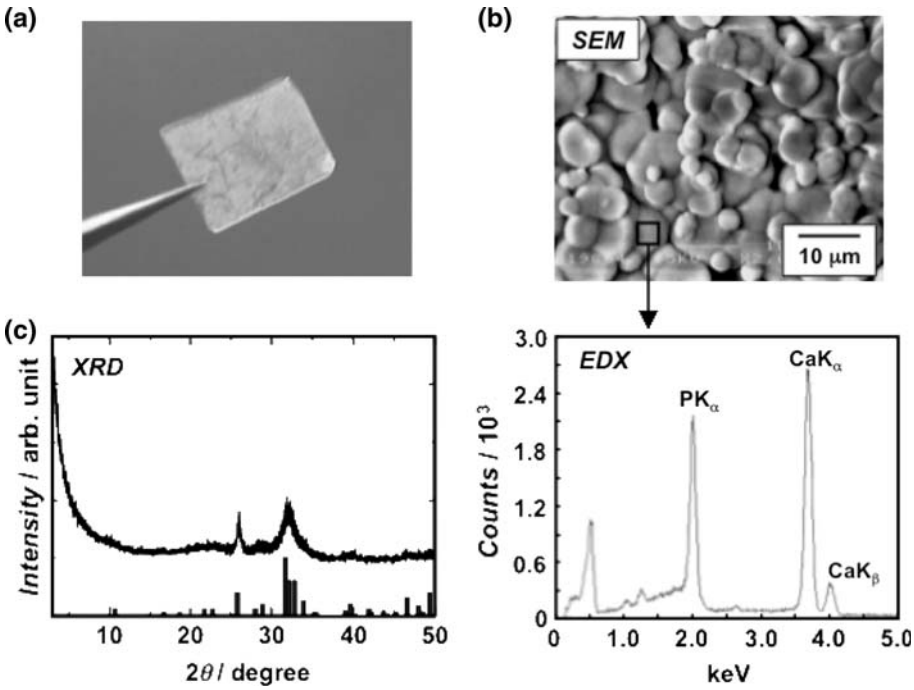

Fig. 2 A macroscopic image (**a**), a SEM image and its EDX spectra (**b**), and the XRD pattern (**c**) of the cerasome **1**-assembled substrate after immersion into 1.5SBF at 36.5°C for 1 week. In the XRD pattern, solid bars are peaks of synthetic HAp from ICDD database (PDF-2) (PDF#09-0432)

carbonated apatite [17]. Moreover, XRD analyses confirmed that these crystals were HAp (Fig. 2c). The XRD pattern of the substrate reveals two distinct peaks at $2\theta = 26°$ and 32°. The reflection at $2\theta = 26°$ is assigned to the (002) diffraction line of HAp, whereas that at $2\theta = 32°$ is an envelope of the (211), (112), and (300) diffraction line of HAp (PDF#09-0432) [35]. Deposition of HAp on the cerasome **1**-assembled surface was comparable to that on the surface of polymers displaying silanol [23–25] or carboxylic acid [25–31] groups.

3.3 Effect of dicarboxylic acid groups on cerasome surfaces on their HAp deposition

We next observed the time dependence of HAp deposition on cerasome-assembled substrates to evaluate effects of **2**—which can display a dicarboxylic acid group on cerasome surfaces—on HAp deposition on cerasomes. Results of SEM and XRD measurements (Fig. 3) and subsequent EDX analysis (data not shown) revealed that cerasome (**1**–**2**) (**2**: 10 mol%) shows much faster HAp deposition

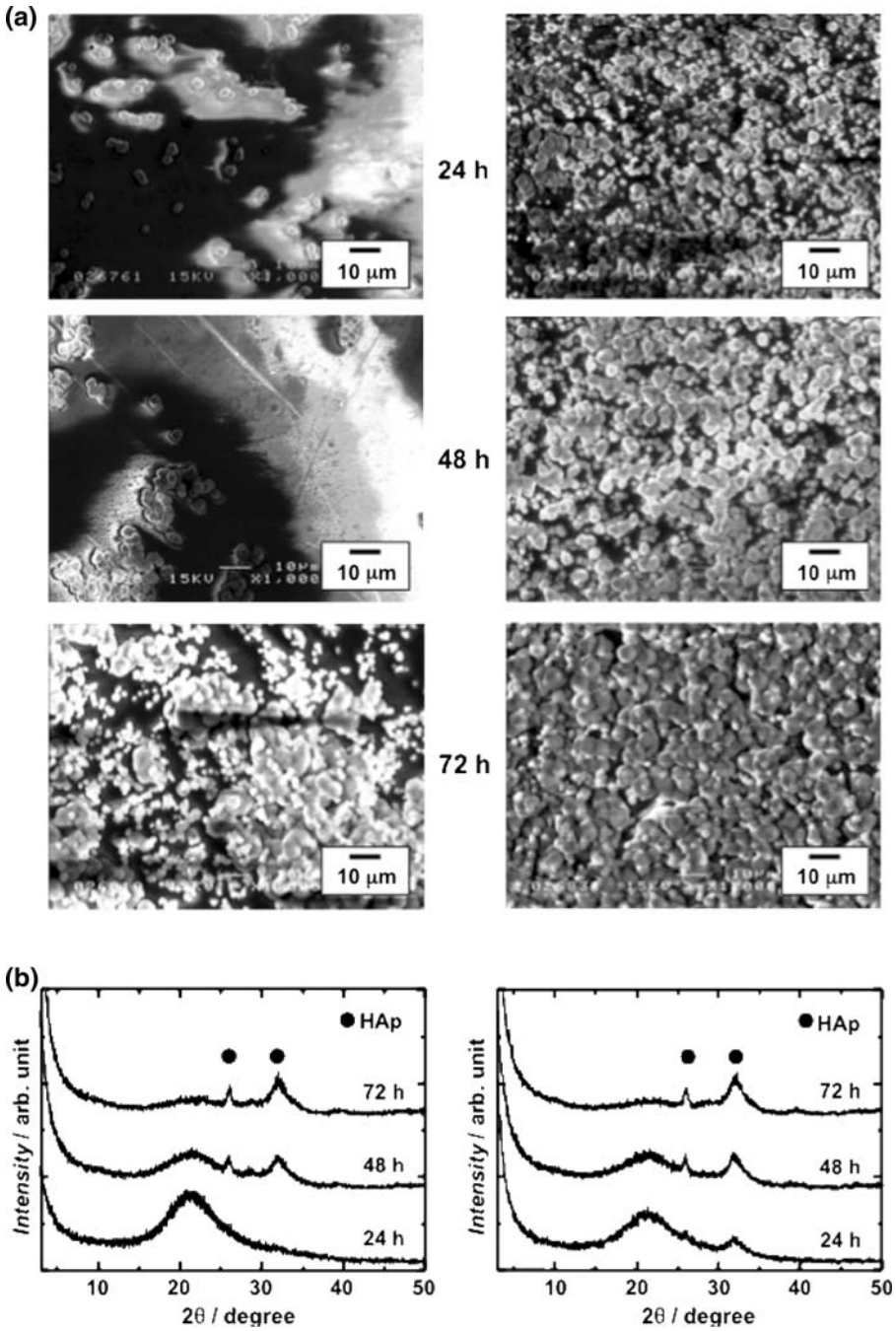

Fig. 3 Time dependence of the SEM images (**a**) and the XRD patterns (**b**) of cerasome **1** and cerasome (**1**–**2**) (**2**: 10 mol%) assembled on glass substrates after immersion in 1.5SBF at 36.5°C. *Left column*, cerasome **1**; *right column*, Cerasome (**1**–**2**)

than that of cerasome **1**. The difference was readily apparent, especially in XRD patterns of the samples after 24 h incubation (Fig. 3b); the peak at 32° that corresponds to HAp crystal appeared in the pattern of cerasome (**1**–**2**), although no such peak was observed in that of cerasome **1**. Longer immersion time (72 h) resulted in complete coverage of both cerasome **1** and cerasome (**1**–**2**) surfaces by thicker HAp layers.

The zeta potentials of the cerasomes in the experimental pH ranges (pH = 7.25) are estimated as about −60 mV for cerasome **1** and as about −40 mV for cerasome (**1**–**2**) from the pH profiles of the zeta-potentials for both cerasomes. The results indicate that the surface charge itself is not a main reason for such a difference in their HAp deposition behaviors. Therefore, as we had expected, the dicarboxylic acid headgroup of **2** probably enhanced calcium ion binding to cerasome surfaces, which increased the HAp crystal nucleation sites. The binding of calcium ion to the headgroup of **2** on the cerasome surface was supported by the fact that cerasome (**1**–**2**) aggregated in aqueous $CaCl_2$ solution and that its D_{hy} turned out to be constant (about 400 nm). Such was not the case of cerasome **1**, as evaluated using DLS (Fig. 4). When dispersions of cerasome **1** or cerasome (**1**–**2**) were added to 1.5SBF, where the final ion concentrations of the resultant solutions were identical to that of SBF, the D_{hy} of both cerasomes increased gradually with increased incubation time (data not shown). The results in the case of SBFs probably reflect several phenomena, such as aggregation of vesicles and deposition of HAp on the vesicular surface. Control of the D_{hy} value is not easy under these conditions. For that reason, further improvements in experimental procedures are necessary to achieve precise nanocoating of HAp onto the surface of the cerasomes that are dispersed in a solution.

Figure 5 portrays the effect of the molar ratio of **2** in the lipids used for preparation of cerasomes on HAp deposition

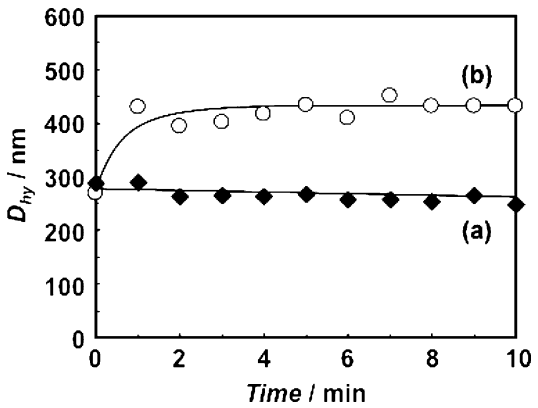

Fig. 4 Time courses of D_{hy} of cerasome **1** (*a*) and cerasome (**1**–**2**) (**2**: 10 mol%) (*b*) in 2.5 mM $CaCl_2$ aqueous solution. [Lipid] = 0.1 mM; 36.5°C

on cerasome (**1**–**2**) surfaces. The SEM and XRD results for cerasomes (**1**–**2**) after 24 h immersion in 1.5SBF at 36.5°C for cerasomes (**1**–**2**) containing more than 5 mol% of **2** showed remarkable enhancement of HAp deposition. In contrast, 0.1 and 1 mol% of **2** in cerasomes (**1**–**2**) were less effective, which indicates that a threshold concentration of **2** exists between 1 and 5 mol% to exhibit remarkable enhancement of HAp deposition on the surface of cerasomes displaying dicarboxylic acid groups.

3.4 Comparison of dicarboxylic acid and monocarboxylic acid groups effects on HAp deposition

To evaluate the superiority of the dicarboxylic acid group, we also examined the effect of molar ratio of **3**, which has monocarboxylic acid headgroup, in cerasomes (**1**–**3**) on HAp deposition in 1.5SBF (Fig. 6). The SEM images and XRD patterns for cerasomes (**1**–**3**) having different molar ratios of **3** after 24 h immersion show that monocarboxylic acid group is also effective to enhance HAp deposition. Results show that **3** also has a threshold concentration between 1 and 5 mol% for the enhancement of HAp deposition. At higher concentrations (>5 mol%) of **2** or **3** in the cerasomes, no difference is apparent between **2** and **3** on the enhancement effect for HAp deposition. On the other hand, at lower concentrations (<1 mol%), **2** more effectively enhances HAp deposition than **3** does (see XRD results for 24 h immersion in Figs. 5b and 6b), which indicates that preorganization of two carboxylic acid group closely together is expected to be more effective for binding of calcium ion, which might become nucleation sites for HAp deposition. At higher concentrations, the headgroups of nascent lipid molecules of **3** can act as a dicarboxylic acid group. For that reason, no large difference was observed between cerasomes (**1**–**2**) and cerasomes (**1**–**3**).

4 Conclusion

Cerasome surfaces induced heterogeneous nucleation and deposition of HAp in 1.5SBF. The deposition was accelerated when carboxylic acid groups were displayed on the cerasome surfaces. Results show that the differences in surface compositions at a molecular level (lipid compositions) amplify mesoscale outputs, i.e. HAp deposition behavior on the surfaces. Such a system presents a new paradigm for fabrication of organic–inorganic hybrid biomaterials in nanoscale and/or mesoscale resolution. The HAp-coated cerasomes can be applied as biocompatible materials, especially for use in bone repair—as carriers for

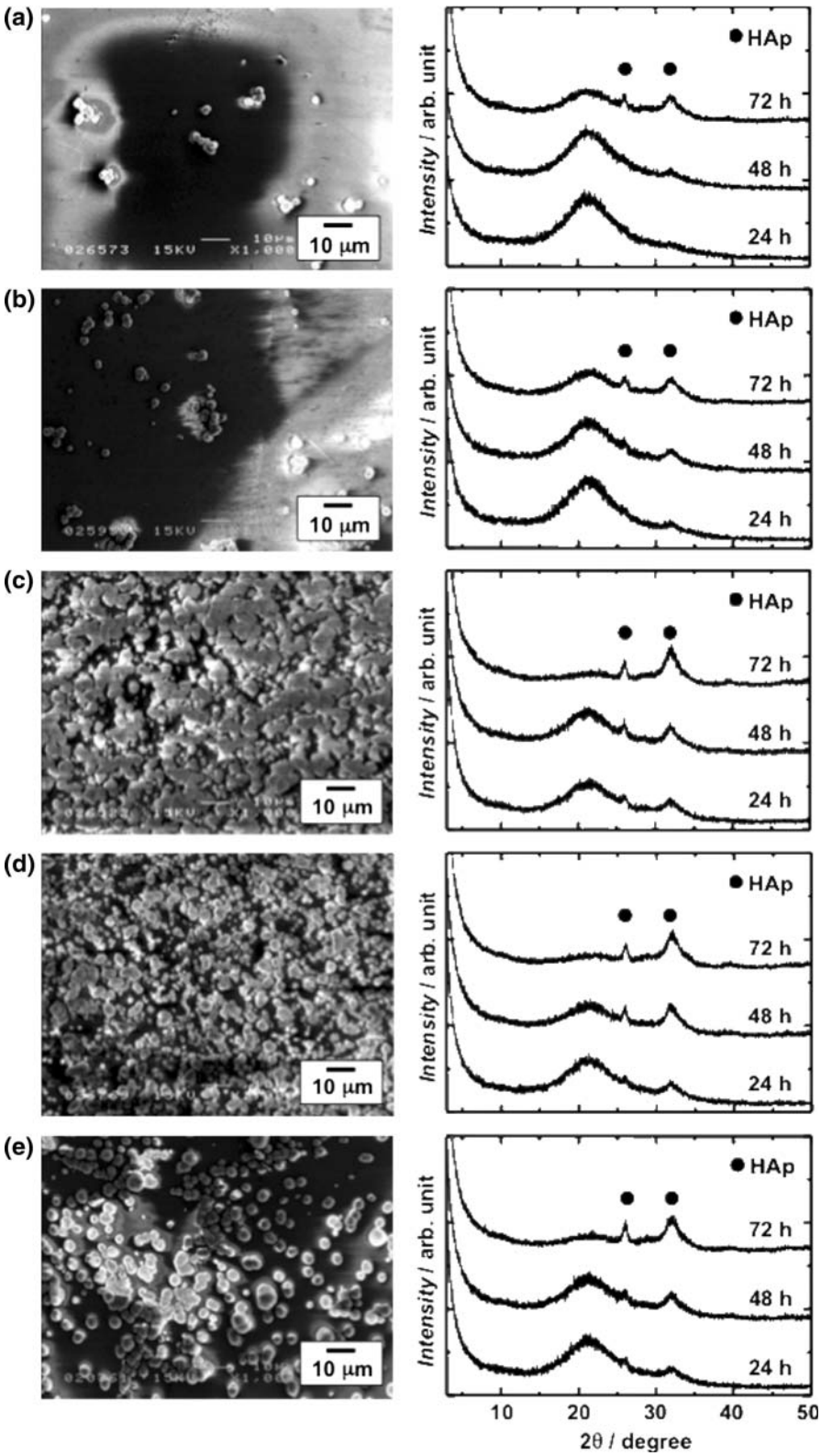

Fig. 5 SEM images (*left*, after 24 h immersion) and XRD patterns (*right*, after 24, 48, and 72 h immersion) of cerasomes (**1**–**2**) assembled on glass substrates after immersion in 1.5SBF. Lipid concentration of **2** for total lipids in the cerasomes: 0.1 (**a**), 1.0 (**b**), 5.0 (**c**), 10 (**d**), and 20 (**e**) mol%

drug delivery systems for bone tissues, and as components of bone implants having material loading ability. The assemblies of HAp-coated cerasomes are also useful in applications such as biocompatible nanosheets and nanoreactors. The flexible lipid bilayer structure of cerasomes further adds functionality to these systems, such as temperature-dependent material release, which is not available using conventional colloidal capsules.

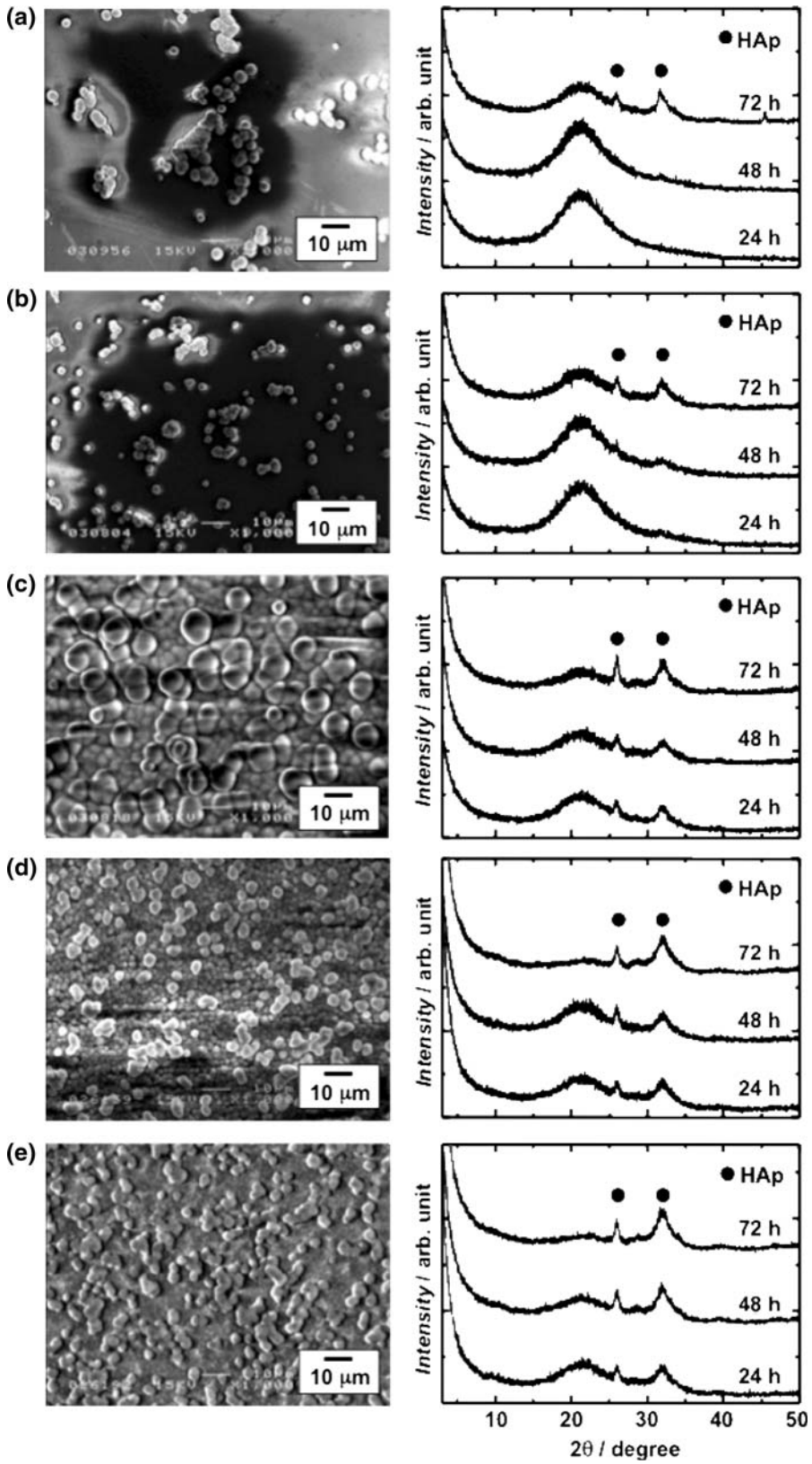

Fig. 6 SEM images (*left*, after 24 h immersion) and XRD patterns (*right*, after 24, 48, and 72 h immersion) of cerasomes (**1**–**3**) assembled on glass substrates after immersion in 1.5SBF. Lipid concentration of **3** for total lipids in the cerasomes: 0.1 (**a**), 1.0 (**b**), 5.0 (**c**), 10 (**d**), and 20 (**e**) mol%

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