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ARTICLE

Fabrication of fibrillized collagen microspheres with microstructure resembling extracellular matrix†

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Microspheres using artificial or natural materials have been widely applied in the field of tissue engineering and drug delivery systems. Collagen is being widely used for microspheres because of their abundancy in the extracellular matrix (ECM), and its good biocompatibility. The purpose of this study is to establish the appropriate condition for preparing collagen microspheres (CMS) and fibrillized collagen microspheres (fCMS) using water-in-oil (W/O) emulsion. Collagen can be tailored to mimic native cell environment possessing similar microstructure to that of the ECM by conditioning the aqueous solution. We focused on the preparation of stable and injectable CMS and fCMS which is stable and would promotes the healing response. Controlling the interfacial properties of hydrophilic-lipophilic balance (HLB) value, we obtained CMS and fCMS with various sizes and various morphologies. The microsphere prepared with wetting agents showed good microsphere formation, but too low or too high HLB value caused low yield and uncontrollable size distribution. The change in the surfactant amount and the rotor speed also affected the formation of the CMS and fCMS, where the low surfactant amount and fast rotor speed produced smaller CMS and fCMS. In the case of fCMS, the presence of NaCl made it possible to prepare stable fCMS without using any cross-linker due to fibrilligenesis and gelling of collagen molecules. The microstructure of fCMS was similar to that to the native tissue indicating the fCMS would replicate its function *in vivo*.

Introduction

Microspheres are widely used in the biomedical field as an effective tool for pharmaceutical applications.[1] Microspheres are fabricated by artificial or natural materials depending on their designated functions or targets. Recently, the use of collagen has brought the new aspects microspheres application because collagen is the major component of the extracellular matrix (ECM) in mammals. Collagen is non-toxic and non-antigenic, and can mimic the natural cell microenvironment. There are numerous methods for preparing collagen microspheres (CMS), such as water-in-oil (W/O) emulsion, oil-in-water (O/W) emulsion method, direct dropcasting of collagen solutions on hydrophobic surfaces, and spraying of collagen solutions onto poor solvents.[2-7]

The main goal of this study was to prepare collagen microspheres (CMS) that can be applied for cell transplantation or cell delivery, especially for targeting normal wound healing while suppressing inflammatory response for plastic and reconstructive surgery. The prominent use of collagen microspheres in the biomedical field is mainly due to its non-toxic and non-antigenic properties as well as good cell adhesibility and good biodegradability. However, actual control of collagen degradability has not been achieved and the question of inflammatory response during its degradation has risen.[8,9] Furthermore, use of cross-linkers to stabilize the CMS still remains in question in terms of encapsulation or toxicity caused by the cross-linker itself. These issues may result in slow recovery, hypertonic scars, or scar contracture, which are the results of an abnormal wound healing process.[10] Normal wound healing without leaving any wound trace behind is most ideal for plastic and reconstructive surgery.

Therefore, we focused on preparing CMS, which degrades slowly and integrates with the surrounding tissues to induce normal wound healing while suppressing inflammatory response. For this purpose, we decided to adopt CMS that consist of fibrillized collagen (fibrillized collagen microspheres; fCMS) with the same microstructure as the native tissue. The idea of preparing fCMS is based on a concept called biorelevant structure-function-property relationship.[11] Considering that the ECM is composed of microlayered structures possessing fibrils (quaternary structure) with regulated *D*-periodicity, we previously constructed a cross-linker-free collagen matrix with a fibrillized structure and found that it degrades much more slowly and suppresses macrophage recruitment.[12] That is, by mimicking the collagen fibril structure of the ECM in the CMS preparation procedure, it is possible to control the properties and functions of CMS. Furthermore, the fibrillization of collagen would bring the stability to the microsphere. There are reports on using non-chemical crosslinking methods to fabricate collagen microspheres. [13-14] However, our method would eliminate any possibility of toxicity and enables the microspheres to be integrated to the surrounding tissue. With this, by establishing the clear result on how the properties of the CMS changes by the preparation condition, it would be easier for us to choose the appropriate CMS for the application experiments.

Here, we report CMS and fCMS preparation using the W/O emulsion technique and establish appropriate preparation conditions for various collagen microspheres. The emulsion technique is the most commonly used method worldwide because of the simple and easy preparation procedure involved. The method is already well established and is widely used not only in research but also in industry. However, it is not yet well established for collagen emulsion. Many studies have reported preparation conditions for CMS or fCMS, but they vary considerably and do not explain why such conditions had to be selected. As the first step for preparing CMS and fCMS, we investigated the relationship between the hydrophilic-lipophilic balance (HLB) value and temperature microsphere formation in W/O emulsion based on the numerous reports on the preparation of CMS by adopting a lipophilic surfactant (Span 80, HLB value 4.3), a wetting agent (Span 20, HLB value 8.6), and a hydrophilic surfactant (Tween 20, HLB value 16.7). Moreover, we prepared a wetting agent of HLB 9.0 by mixing Span 20 and Tween 20 to determine the effect of further decreasing the interfacial tension between water and oil.[2, 4-5, 7, 15-16]

Experimental

Materials

Collagen type I solution (0.5 wt%; pH 3) was purchased from KOKEN (Tokyo, Japan). Liquid paraffin, sorbitan monooleate (Span 80, HLB value 4.3), sorbitan monolaurate (Span 20, HLB value 8.6), Polysorbate 20 (Tween 20, HLB value 16.7), water-soluble carbodiimide [N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide, WSC], and sodium chloride (NaCl) were purchased from Wako Pure Chemical Industries (Tokyo, Japan) and used without further purification.

Methods

Preparation of collagen microspheres (CMS). Ten milliliters of 1% (w/v) collagen solution was mixed with 1 mL of water containing 50 mg of WSC. The solution was dropped into 50 mL of liquid paraffin containing 1% (v/v) Span 20 that was being stirred at 600 rpm using a mechanical stirrer (BL600, SHINTO Scientific Co. Ltd., japan) at room temperature. The emulsion process was performed for 30 min. Then, 1 mL of water containing 250 mg WSC was added to the emulsified mixture and stirred for extra cross-linking. After 30 min, 50 mL of ethanol/water mixture (50/50, v/v) was added into the emulsion and mixed for 5 min to rinse the CMS. The mixture was centrifuged at 3500 rpm for 5 min to separate the solution from the CMS. This procedure was repeated 3 times to remove oil. Water (MilliQ®) was added to the CMS and mixed; the mixture was centrifuged at 3500 rpm for 5 min to remove the residual ethanol to the maximum extent possible. This procedure was repeated twice. Then the CMS was transferred to fresh water and kept at room temperature. This experiment was repeated using 1% Span 80 and Tween 20 as the surfactant. Furthermore, a surfactant possessing HLB value of 9.0 was prepared by mixing Span 20 and Tween 20 before putting into the oil using the following equation:

$$\mathrm{A} = \frac{X - HLB_{\mathrm{B}}}{HLB_{\mathrm{A}} - HLB_{\mathrm{B}}} \times 100 \quad (1)$$

$$\mathrm{B}\ (\%) = 100 - \mathrm{A}\ (\%) \quad (2)$$

where A (%) and B (%) represent Span 20 and Tween 20, respectively. *X* is the targeted HLB value.

To prepare size-controlled CMS by changing the surfactant concentration and the rotor speed, respectively, the collagen solution was dropped into the liquid paraffin containing surfactant (0.5, 1, or 2%) which has been controlled at HLB = 9.0 using equation 1. The rotor speed was fixed at 600 rpm. Furthermore, the effect of rotor speed was investigated by stirring the liquid paraffin at 300, 600, 900, or 1200 rpm, while fixing the surfactant concentration at 1 wt%.

Preparation of fibrillized collagen microspheres (fCMS). Five milliliters of 0.5% (w/v) collagen solution was mixed with 5 mL of water containing 1.8% NaCl (w/v) to obtain 0.9 wt% NaCl collagen aqueous solution. The solution was dropped into 50 mL of liquid paraffin containing 1% (v/v) surfactant that was being stirred at 600 rpm at 25°C, 30°C, or 37°C using a mechanical stirrer for 1 h to prepare fCMS, respectively. After 1 h, 50 mL of ethanol/water mixture (50/50, v/v) was added into the emulsion and mixed for 5 min to rinse the fCMS. The mixture was centrifuged at 3500 rpm for 5 min to separate the solution from the precipitated fCMS. This procedure was repeated three times to remove oil. Water (MilliQ®) was added to the fCMS and mixed; the mixture was centrifuged at 3500 rpm for 5 min to remove the residual ethanol to the maximum extent possible. This procedure was repeated twice. Then, the fCMS was transferred to fresh water and kept at room temperature. fCMS was prepared using a surfactant with an HLB value of 4.3, 8.6, 9.0 and 16.7, respectively. The effect of surfactant concentration and rotor speed was investigated by controlling the surfactant concentration (0.5, 1, or 2%) and rotor speed (300, 600, 900, or 1200 rpm).

Observation of the CMS and fCMS. Images of CMS and fCMS after the last rinse with water were taken with a phase contrast microscope (TE2000-U, Nikon, Tokyo, Japan) to observe the shape of the CMS. The diameter of microspheres was measured directly using a scale of at least 100 microspheres. To observe the microstructure of CMS and fCMS, they were air-dried overnight after dropping CMS or fCMS suspension on the scanning electron microscope (SEM) sample stand with carbon tape on it before coating with gold (SC-701AT, Sanyo Denshi, Tokyo, Japan). Then, they were observed with an SEM (S-3400NK, Hitachi) for their shape and surface characteristics. For analyzing *D*-periodicity, the surface of the samples was observed using an atomic force microscope (AFM, SPA300, Seiko Instruments, Chiba, Japan). After dropping the microsphere suspension on the Cell Desk® (Sumitomo Bakelite, Tokyo, Japan), the water was blown off using a dust blower. Fibril band formation was observed using AFM with a silicon micro-cantilever (SI-AF01, Seiko Instruments Inc., Chiba, Japan). The structures of the collagen fibrils observed with AFM were all compared with those reported in previously published articles.[17-19] Simple coating of the collagen fibrils on the surface of the polymer substrate was executed by diluting the aqueous collagen solution in saline (1:100) before dropping it onto the Cell Desk® and leaving it overnight at room temperature.

Deformation evaluation of CMS and FCMS by syringe extrusion via needle. The CMS and FCMS aqueous solution was put into the syringe with 27G needle. Then the syringe was fixed firmly at the syringe pump before extruding the aqueous solution at the speed of 50mL/min. To calculate the pressure applied to the CMS and FCMS, the Poiseuille's Law was adopted for defining the rate of flow of volume q of a viscous fluid through a cylindrical channel the was adopted, assuming the flow in the needle is laminar;[20,21]

Table 1. Effect of preparation conditions, size distribution, variation coefficient, and yield percentage of respective CMS. Bold italic numbers represents the altered variation.

HLB value	Collagen concentration (in saline, %)	Surfactant concentration (%)	Rotor speed (rpm)	Size range (μm)	Average diameter (μm)	Variation coefficient	Yield (%)
4.3	1.0	1.0	600	2.2-440	50.3 ± 60.1	1.19	40
8.6	1.0	1.0	600	2-90.3	13.0 ± 12.0	0.93	60
9.0	1.0	1.0	600	2.1-49.3	9.0±6.8	0.8	70
16.7	1.0	1.0	600	59.7-314.5	192.4 ± 50.0	0.26	70

$$q = \frac{\pi r^4}{8\eta}\frac{\Delta P}{\Delta L} \quad (3)$$

where r is the radius of the channel (needle), h is the viscosity of the fluid (water), ΔL is the length of the channel, and ΔP is the pressure difference between the ends of the channel (internal pressure - atmospheric pressure). The samples were re-collected and observed with phase contrast microscope.

Cell culture in vitro. Cell culture experiment of NIH3T3 using the CMS and fCMS was executed. The CMS and fCMS was dispersed in saline and was sterilized using a high hydrostatic pressure device (Dr.CHEF, Kobe Steel, Japan) by applying high hydrostatic pressure (5000 atm) at 10°C for 5 minutes. Then 50 μL CMS and fCMS suspensions was added dropwise to the non-treated 96well culture plate (polystyrene, Falcon, USA) having 10% Fatal bovine serum (Life Technologies, USA), 100U/mL Penicillin (Sigma, USA), and 100μg/mL Streptomycin (Life Technologies, USA) containing Dulbecco's Modified Eagle Medium (Life Technologies, USA). In addition, this suspension was prepared to the extent that the bottom of each well is filled with fine particles. After 15 minutes, a suspension of NIH3T3 (500 cells/well) were added in 150μL to each well, and cultured for 7 days. The live and dead assay was executed using 2 μg / mL Calcein-AM / PI solution on day 1 and day 7.

Fabrication of 3D matrix of CMS and fCMS. After sterilizing the CMS and fCMS with high hydrostatic pressure device, CMS and fCMS suspension was put into the cell culture insert (Inserts for 12-well plates, Falcon, USA) having NIH3T3 (3000 cells/mL) in the cell culture medium (1mL). The cell culture was continued for 14 days. Then, the tissue-like structure was taken out for staining cross sections with hematoxylin-eosin. These matrices were fixed by 10% formalin, dehydrated stepwise using ethyl alcohol, immersed in xylene, and embedded in paraffin. Paraffin sections (4 μm) were cut and stained with hematoxylin-eosin.

Results and discussion

Here, the concentration of the surfactant was fixed to 1 wt% as the standard condition, and the HLB value of the surfactant was controlled at 4.3, 8.6, 9, and 16.7 to investigate the effects on CMS formation. Despite the HLB value, all CMS were round-shaped with a heterogeneous size distribution as shown in optical microscope (Fig 1a to 1d) and scanning micron electroscope (SEM) (Fig 1e to 1h). The dispersant was in a clear transparent liquid form and remained transparent for more than a month (Fig S1a and S1c). An increase in the HLB value of the surfactant results in a decrease in the microsphere size (HLB value, 4.3–9). The average CMS size was low when a wetting agent was used, although the variation coefficient was large (Table 1). The yield percentage was the lowest (40%) when Span 80, which has an HLB value of 4.3, was used. This low yield is due to strong interfacial barriers formed by highly lipophilic surfactants. Rinsing had to be repeated and the resulting yield percentage was lower than that of any other surfactants. The formation of smaller microspheres is thought to be due to the decrease in the interfacial tension caused by the wetting agent. It is known that decreasing the surfactant tension causes a decrease in the size of the droplet in the continuous phase when all other variables remain constant.[1] Furthermore, it was easier to rinse the CMS using an ethanol/water mixture (50/50) and then water and a better yield percentage was obtained than that observed with HLB=4.3. Stabilization was achieved by cross-linking the CMS with WSC. The cross-linking procedure had to be repeated, since the cross-linking rate was low when WSC was used as the cross-linker in water. This is because the fast hydrolysis rate of

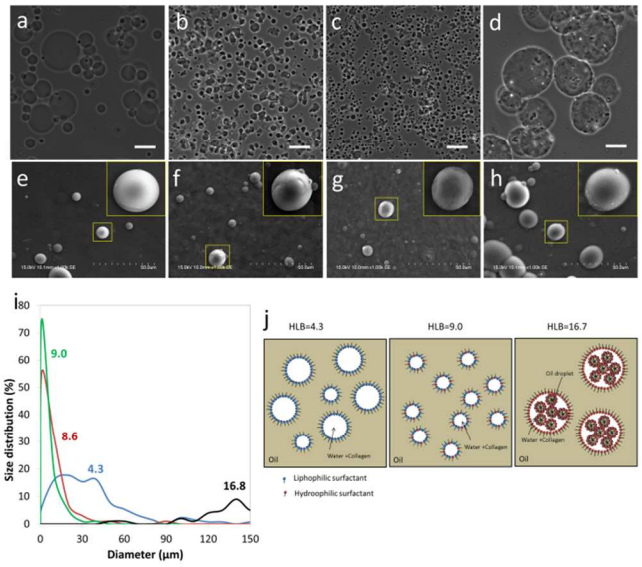

Fig 1. Photographs (upper, bar = 100 μm) and SEM images (below) of collagen microspheres prepared using surfactants with HLB values of 4.3 [(a) and (e)], 8.6 [(b) and (f)], 9.0 [(c) and (g)], and 16.7 [(d) and (h)]. SEM images in the large frame are shown at a magnification of 1000× the small frame. (i) Proportion of CMS size prepared by surfactants with HLB values of 4.3 (a), 8.6 (b), 9.0 (c), and 16.7 (d). (j) Schematic image of CMS preparation under various HLB conditions.

the WSC in water.[16] When Tween 20 was used sudden increase in the CMS size was observed. Tween20, is hydrophilic surfactant (HLB value 16.7) that is used for oil-in-water (O/W) emulsion. The oil droplet is formed in the aqueous collagen

layer, which is surrounded by the oil. This resulted in formation of oil-in-water-in-oil (O/W/O) emulsion causing increase in the average size (ϕ = 207μm) (Fig 1 d and Fig 1 j).

Figure 2 shows the results of CMS formation according to the change in the surfactant concentration and the rotor speed. As expected, the size of the CMS changes according to the amount of surfactant present.[1] A higher amount of surfactant resulted in smaller CMS, which matched the results previously reported by other researchers. However, we did not notice significant differences when 0.5 and 1 wt% were used. Furthermore, no CMS formed below 0.5 wt% because the coalescence of aqueous droplets in the continuous oil phase is reduced by surfactants, and high surfactant concentrations do not alter the interfacial tension once the critical micelle concentration (CMC) is reached.[22] Therefore, the surfactant concentration should be higher than 0.5 wt% in this case. When the rotor speed was increased to 1,200 rpm, small CMS were obtained (Fig 2b). Size heterogeneity occurred when a low rotor speed was used. This phenomenon was expected as higher speeds induce a good homogenizing effect produced by stronger shear force and increased turbulence, reducing the size of the water droplets in the oil.[23]

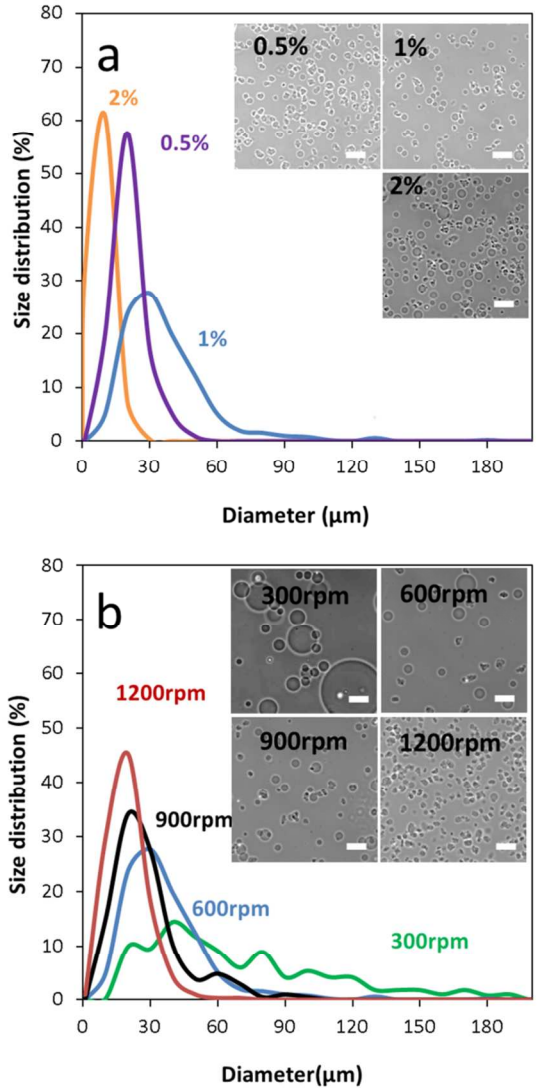

Fig 2. The size distribution and the photographs of CMS (upper, bar = 100 μm) prepared by altering surfactant concentration (0.5% – 2%) (a) and by changing the rotor speed from 300 rpm to 1,200 rpm (b). The rotor speed was set at 600 rpm for (a) and the surfactant concentration was set at 0.5% for (b) (HLB = 9.0).

To obtain CMS with fibrillized structure, fibrillization of collagen was executed by controlling the NaCl concentration of the aqueous solution. The collagen molecules align with certain regularity when NaCl is added to the aqueous solution, causing fibrillogenesis. The collagen alignment that is seen in native tissue can be obtained when collagen encounters a physiological environment. Fibrillogenesis is an entropy-driven process caused by electrostatic and hydrophobic aggregation between the collagen molecules.[19] They induce collagen alignment, eventually causing the formation of collagen fibrils with *D*-periodicity. At a NaCl concentration of 0.9 wt%, the collagen molecules are aligned to form fibrils that most closely resemble those of the native ECM.[24-26] However, the problem was whether this phenomenon could be obtained in W/O emulsion.

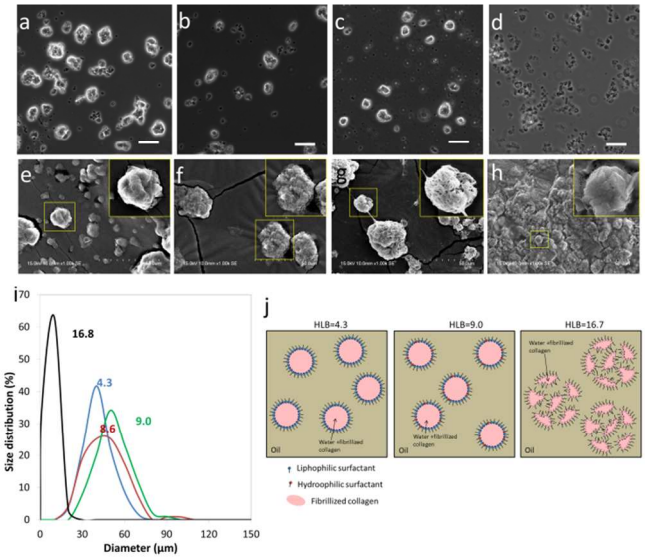

Fig 3. Photographs of fibrillized collagen microspheres (upper, bar = 100 μm) and SEM images (below) prepared by surfactants with HLB values of 4.3 [(a) and (e)], 8.6 [(b) and (f)], 9.0 [(c) and (g)], and 16.7 [(d) and (h)]. SEM images in the large frame are shown at a magnification of 1000× the small frame. (i) Proportion of fCMS size prepared by surfactants with HLB values of 4.3 (a), 8.6 (b), 9.0 (c), 16.7 (d). (j) The schematic image of fCMS preparation under various HLB conditions.

Fig 3 shows the results of fCMS formation with various surfactant HLB values. Generally, the shape of the fCMS was round as shown with optical microscope and scanning electron microscope (Fig 3a to 3h). The average size of the fCMS varied according to the preparation conditions, but the sizes were all

Table 2. Effect of preparation conditions, size distribution, variation coefficient, and yield percentage of respective fCMS. Bold italic numbers represents the altered variation.

HLB value	Collagen concentration (in saline, %)	Surfactant concentration (%)	Rotor speed (rpm)	Size range (μm)	Average diameter (μm)	Variation coefficient	Yield (%)
4.3	0.5	1.0	600	2.7-40.5	13.5 ± 10.1	0.75	20
8.6	0.5	1.0	600	3.5-73.4	26.0 ± 19.34	0.74	40
9.0	0.5	1.0	600	31.8-96.5	56.9±12.1	0.2	40
16.7	0.5	1.0	600	3.5-27.6	11.7 ± 3.9	0.30	80

below 50 μm. When the size distribution was observed, it was found that the size of the most abundant fCMS had not changed compared to that of the CMS, where more than 50% of microspheres were below 20 μm in diameter (Fig 3i and Table 2). In the case of Tween 20, we did not observe the formation of round-shaped fCMS because the O/W/O had formed hollow fCMS, which had been destroyed by the mechanical shear stress caused by the rotor (Fig 3j). This resulted in aggregation during the sample preparation for SEM (Fig 3h). Good stabilization after CMS and fCMS formation could be observed. The fCMS precipitated whereas CMS remained dispersed in water or in PBS (Fig S1b and S1d). For CMS, the stabilization was achieved by addition of WSC twice during the procedure. Although no cross-linkers were used for fCMS, we did not observe changes in size or number, or any signs of burst, aggregation, or flocculation after 1 month in water or PBS. We also applied the extrusion pressure to the CMS and fCMS in the 10mL syringe to evaluate the stabilization upon pressure using 27G (ϕ=0.21mm^2) at 50 mL/min. The change in the shape of the CMS and fCMS after the extrusion was not shown (Fig S2). Furthermore, no flocculation or breakdown of the CMS or fCMS could be observed after 1 week in water or in PBS. The pressure internal flow applied to the CMS and fCMS in the needle was 350kPa (see equation 3), which is above the high injection pressure region (39.9 – 99.7kPa).[27] This indicates that no deformation to the CMS or fCMS would occur during normal injection procedure. For CMS, the cross-linker had provided the stability to the microsphere while the fibrillogenesis and gelling effects had provided stability to the fCMS. To our knowledge, this is the first study where no cross-linker was used for preparing collagen microspheres. Not using any cross-linker is beneficial in two aspects; the toxicity caused by the chemical components can be avoided, and the degradation of the collagen microspheres by the collagen rearrangement instead of collagen cleavage occurs, which suppress the inflammatory response. It should be noted that the batch-to-batch reliability is high in our experiment. We had to be sure that the reproducibility is high. This is because NaCl is being used in our study and we did not know whether the same fCMS would be produced every time. The result in Fig S3 shows that the no significant difference among the batches tested in this experimental system, indicating high reliability of our experiment.

The problem with the preparation of fCMS is the timing for adding the NaCl to the collagen aqueous solution, how to keep the NaCl in the aqueous condition, and the temperature during the dispersion in the oil phase. Here, NaCl was added directly to the collagen solution to prepare a 0.9 wt% NaCl collagen solution before dropping it into the oil (25°C, 30°C, or

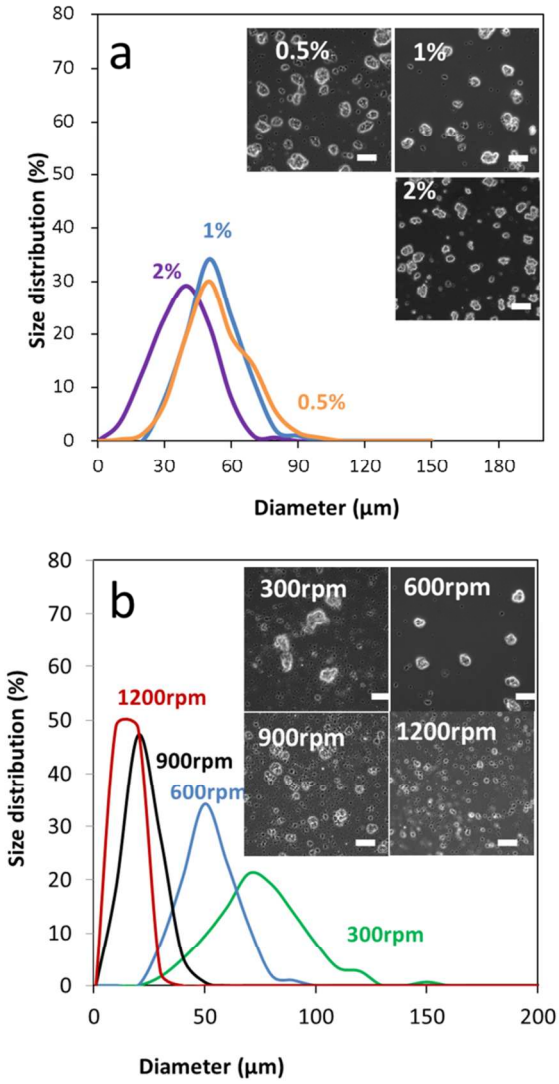

Fig 4. The size distribution and the photographs of fCMS (upper, bar = 100 μm) prepared by altering surfactant concentration (0.5% – 2%) (a), and by changing the rotor speed from 300 rpm to 1,200 rpm (b). The rotor speed was set at 600 rpm for (a) and the surfactant concentration was set at 0.5% for (b) (HLB = 9.0).

Table 3. Effect of surfactant concentration and rotor speed on the diameter and the dispersity of the fCMS. Bold italic numbers represents the altered variation.

HLB value	Collagen concentration (in saline, %)	Surfactant concentration (%)	Rotor speed (rpm)	Size range (μm)	Average diameter (μm)	Variation coefficient	Yield (%)
9.0	0.5	***0.5***	600	19.7–107	58.4 ± 14.5	0.25	55
9.0	0.5	***1.0***	600	31.8–96.5	56.9 ± 12.1	0.21	40
9.0	0.5	***2.0***	600	14.1–88.3	43.4 ± 13.0	0.3	38
9.0	0.5	1.0	***300***	12.8–152.6	79.2 ± 20.2	0.26	50
9.0	0.5	1.0	***900***	12.9–52.9	27.5 ± 7.65	0.28	30
9.0	0.5	1.0	***1200***	11.3–39.4	20.5 ± 4.28	0.21	35

37°C, respectively). The temperature had to be 30°C for the successful preparation of fCMS because at 25°C, the fCMS were unstable and phase separation had occurred afterward. On the other hand, at 37°C, we could not obtain spherical microspheres and only chunks of collagen gels were visible after rinsing (Fig S4). This result implies that the gelling procedure is necessary as the hardening procedure for the stabilization of fCMS. A temperature that is too low would not induce the gelling effect, and a temperature that is too high would result in gelling that occurs too quickly, indicating that a balance between fibrillization and gelling should be achieved.[25] This can be supported by the fact that all 3 samples possesses fibrillized structure (Fig S4, below). That is, the simple fibrillogenesis does not bring stabilization effect and stabilization procedure is essential. The gelling effect is the result of partial denaturation of the collagen due to the temperature or loosening of the helices.[28] Therefore, the temperature should be controlled to provide collagen microspheres enough time to form water/collagen droplets in oil (liquid phase). Fibrillogenesis should occur simultaneously with gelling to form stable fCMS (solid phase). It is thought that the balance between gelling and fibrillogenesis can be achieved at 30°C.

We expanded our study to find whether the formation of fCMS would occur by altering surfactant concentration and rotor speed at HLB=9.0 as shown previously with CMS (Fig 4 and Table 3). An increase in the surfactant concentration and the rotor speed resulted in a decrease in fCMS size. The rule generally followed that of CMS, but the average size was slightly higher than that of CMS (Fig 4 and Table S1). A higher amount of surfactant resulted in smaller CMS, but, we did not notice significant differences when 0.5 and 1 wt% of surfactants were used. Furthermore, no CMS formed below 0.5 wt% because the coalescence of aqueous droplets in the continuous oil phase is reduced by surfactants, and high surfactant concentrations do not alter the interfacial tension once the critical micelle concentration (CMC) is reached.[22]

Furthermore, we observed that the variation coefficient was lower than that of CMS, implying that a very homogeneous size distribution can be obtained for fCMS. The high variation coefficient at low rotor speeds indicates that a minimum speed is required to produce homogeneously-sized CMS or fCMS. The smallest size of fCMS was observed on preparation at a rotor speed of 1,200 rpm. The small size of the fCMS is not the result of the breakdown of the larger fCMS as shown when a hydrophilic surfactant (HLB = 16.7) was used. Size heterogeneity occurred when a low rotor speed was used. This phenomenon was expected as higher speeds induce a good homogenizing effect produced by stronger shear force and increased turbulence, reducing the size of the water droplets in the oil.[21] The main disadvantage of this system is the low yield percentage. As shown in Table 3, we could also observe that the yield was higher for CMS than fCMS for the all conditions, where the highest yield percentage for fCMS was only 55%. It is not clear at this point why the yield percentage is higher for CMS, but we think that this is because of the efficiency in collagen chain rearrangement. As mentioned before, the stabilization of fCMS is obtained by gelling/fibrillogenesis.[12] Therefore, CMS which did not turn into the fCMS has naturally

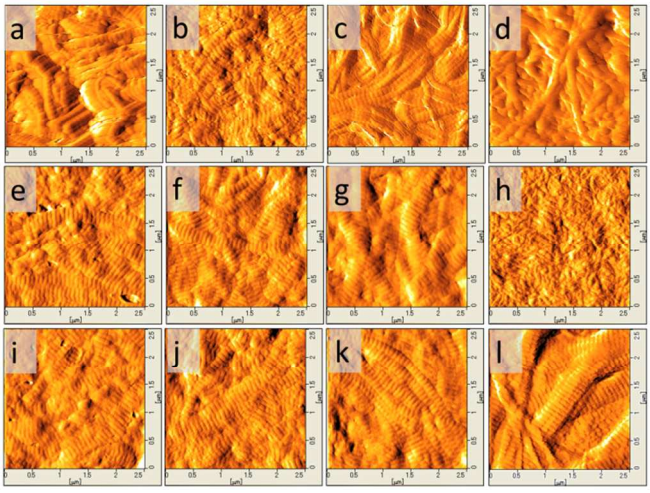

Fig 5. Deflection AFM images of fCMS prepared by surfactants with various HLB values (upper), rotor speeds (middle), and surfactant concentrations (below). HLB control: (a) 4.3, (b) 8.6, (c) 9.0, and (d) 16.7. Rotor speed control: (e) 300, (f) 600, (g) 900, and (h) 1,200 rpm. Surfactant concentration control: (i) 0.5%, (j) 1%, and (k) 2%. (l) Native collagen fibrils prepared on the polymer substrate in 0.9 wt% NaCl.

lost its microsphere form due to instability. Comparing the yield percentage of CMS and fCMS, the yield percentage of fCMS is roughly 44 to 92% of CMS (except for fCMS prepared in HLB=16.7). This implies that of the CMS which is forming W/O emulsion, the approximately 63 ± 16 % of the CMS turns into fCMS (See Table S2 for the calculation).

The fibrillized structure was confirmed using atomic force microscopy (AFM; Fig 5). As mentioned before, we could not be sure that fibrillogenesis of collagen can be formed in W/O emulsion. But, we could observe the fibrillized structure with fibril bands on the surface when a surfactant in the HLB range of 4.3–9.0 was used. The *D*-periodicity, which is the typical collagen fibril morphology, was found to be approximately 72 nm, that is, slightly longer than that of the native tissue. We also observed the formation of thick fibrils, which suggested that lateral assembly of fibrils had occurred, indicating the assembly and fusion of individual fibrils in emulsion.[24] The difference between the CMS and fCMS could be clearly observed: a smooth surface was seen for CMS (Fig S5). The appearance of the fibril band for fCMS confirms the NaCl-triggered alignment of collagen molecules leading to the formation of the collagen fibrils with a microstructure resembling that of the native tissue.[12,26] When Tween 20 was used, fibrils were observed, but we did not observe any fibril band as was seen for other samples. This is due to collagen aggregation in the limited space by the aggregated O/W/O collagen emulsion with collagen surrounding the oil droplet, causing it to become the gel phase before the fibrillization is completed. As mentioned above, the CMS/fCMS yield ratio was almost the same, due to the breakdown of the CMS. Another exception was observed for preparation using a rotor speed of 1,200 rpm. The fibrils were very thin but clear images of fibrils, which are different from those of CMS, were observed on topographic images of AFM (Fig S6). The small size of the fCMS might have caused faster gelling than that seen for fibrillogenesis, not providing enough time for the fibrils to grow before stabilizing.

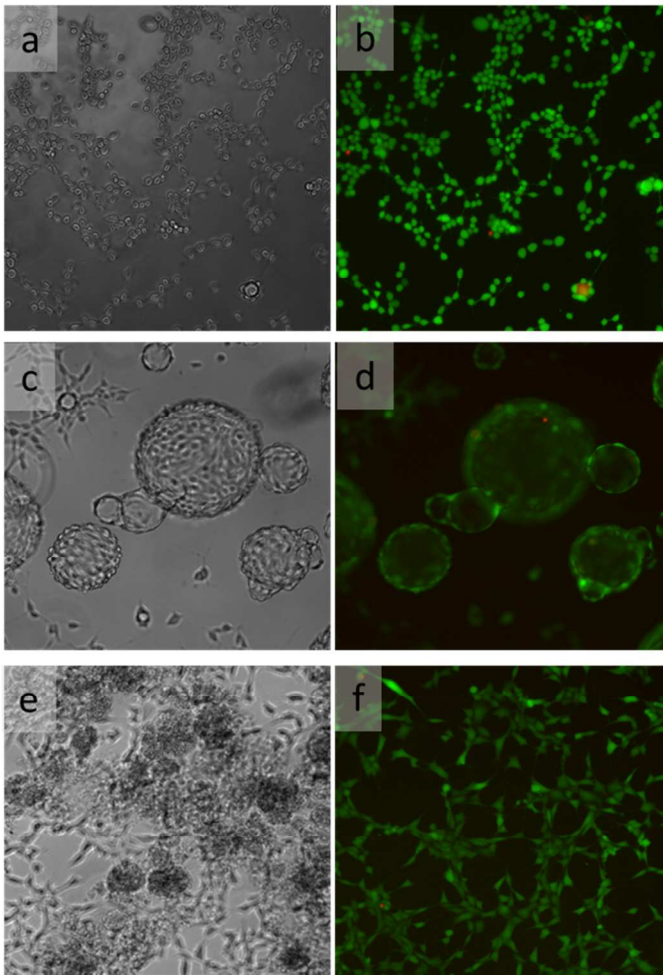

Fig 6. The cellular behaviour of non-treated surface (a) and (b), CMS (c) and (d), and fCMS (e) and (f) after 7 days. The Right images are the fluorescent images of cells (magnification ×10).

The cell-CMS and cell-FCMS interaction *in vitro* is shown in Figure 6. The cells have adhered onto the CMS showing round shape of CMS, while the cells formed network around the fCMS after 7 days of culture. We think that cells did adhere onto the fCMS because the cells have proliferated and changed the shape forming network-like structure. It is thought that the morphological difference between the CMS and fCMS have affected the cell adhesion. That is, the cells adhere weakly on the fibrillized surface. It should be noted that the cells on the fCMS is also different form that from non-adhesive surface where the cells were round shape and did not for any network. This means that the cells are proliferating on the fCMS by weak adhesion. The enlargement of the CMS after 7 days is the characteristics of CMS, where CMS tends to be bigger in PBS at 37°C while fCMS did not show any change in the size (Figure S7). It is not clear at this point why the change in the size had occurred for CMS.

When the cell culture in the fCMS was expanded to 14 days, the formation of the large tissue-like structure had occurred. When the H-E staining was executed, we could observe the cells exist among the fCMS forming network (Figure 7). This implies that the 3-dimensional cell culturing had been achieved. The distribution of the cells was uniform and there was no sign of cell infiltration into the fCMS. It should be noted that the aggregation of the CMS did not occur. As mentioned above, the CMS tend to increase in size but there is not interaction between the CMS. For fCMS, weak cell-fCMS interaction and the cell proliferation had formed network using fCMS as scaffold to create a tissue-like structure in 3-dimension. Figure

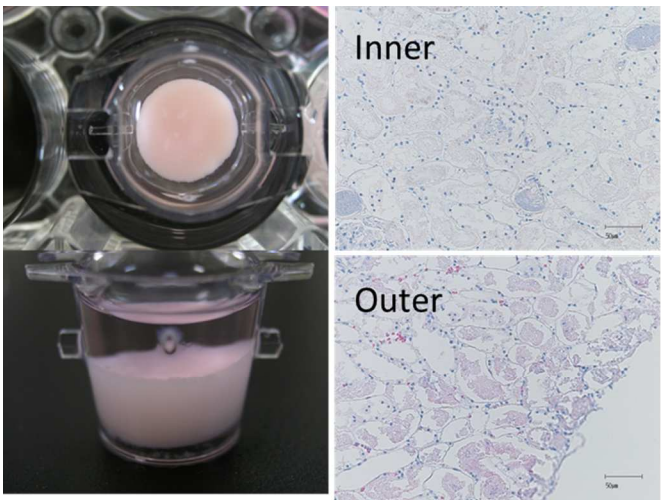

Fig 7. The photographic images (left) and HE stained images (right) of tissue-like structure after 2 weeks incubated with fCMS. The cellular network eventually formed an artificial tissue using fCMS as the scaffold.

7 image is very similar to that can be seen in the healing process, where the cells infiltrated into the implant. It is expected that if the fCMS is injected into the living body with the cells, the self-aggregation would occur but the cells infiltrated between the fCMS promoting the integration between the tissue and fCMS like shown in Figure 7.

Conclusions

We have successfully produced fCMS resembling the collagen fibril microstructure of native tissues by altering various preparation conditions. fCMS is more like a collagen particle that is stabilized by fibrillogeneis and gelling. Since no cross-linkers are used, it is expected that degradation would occur *in vivo* without causing chronic inflammatory responses or encapsulation during the drug release process. Our method also showed high batch-to-batch reliability and reproducibility. To our knowledge, this is the first study to report the preparation of stable fibrillized collagen microspheres that possess a structure similar to that of the native extracellular matrix by using W/O emulsion in low rotor speed (600 rpm). We have found how the morphology change affects the cellular behaviour in vitro. The cellular network formed on the fCMS made it possible to create a 3-D tissue-like structure using fCMS as scaffold. We are investigating the cellular behaviour of fCMS *in vitro* and *in vivo*, and will be reporting its results in the near future.

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Notes and references

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† Electronic Supplementary Information (ESI) available: photographic images of CMS and fCMS; optical microscope images of fCMS before and after pressurization; AFM images of CMS (deflection mode) and fCMS (topological mode); Tables of CMS and fCMS values. See DOI: 10.1039/b000000x/

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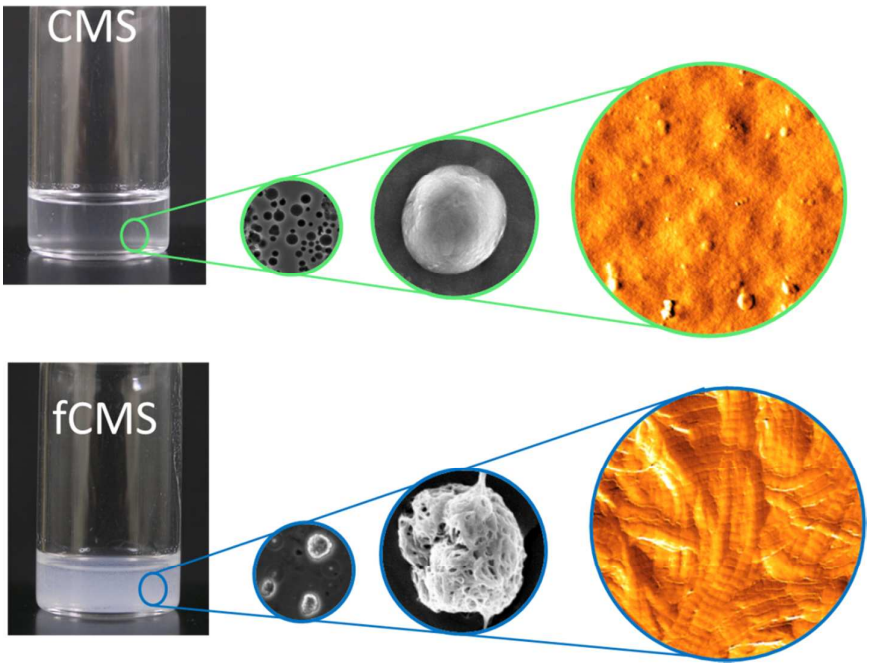
A graphical and textual abstract



By controlling the interfacial properties of hydrophilic-lipophilic balance (HLB), surfactant concentration, rotor speed, inorganic salt, and temperature, fibrillized collagen microspheres (fCMS) possessing the same fibril structure as that of native tissue were prepared using water-in-oil (W/O) emulsion.

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