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Preparation and characterization of collagen microspheres for sustained release of VEGF

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Abstract In this study, we prepared injectable collagen microspheres for the sustained delivery of recombinant human vascular endothelial growth factor (rhVEGF) for tissue engineering. Collagen solution was formed into microspheres under a water-in-oil emulsion condition, followed by crosslinking with water-soluble carbodiimide. Various sizes of collagen microspheres in the range of 1–30 μm diameters could be obtained by controlling the surfactant concentration and rotating speed of the emulsified mixture. Particle size proportionally decreased with increasing the rotating speed (1.8 μm per 100 rpm increase in the range of 300–1,200 rpm) and surfactant concentration (3.1 μm per 0.1% increase in the range of 0.1–0.5%). The collagen microspheres showed a slight positive charge of 8.86 and 3.15 mV in phosphate-buffered saline and culture medium, respectively. Release study showed the sustained release of rhVEGF for 4 weeks. Released rhVEGF was able to induce capillary formation of human umbilical vein endothelial cells, indicating the maintenance of rhVEGF bioactivity after release. In conclusion, the results suggest that the collagen microspheres have potential for sustained release of rhVEGF.

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1 Introduction

Recent studies report that angiogenesis is useful for tissue engineering applications, especially for large tissue formation. Vascular endothelial growth factor (VEGF) has been reported as a potent angiogenic molecule [1]. However, VEGF administered in the body can be degraded and cleared from the body within short time periods, leading to repeated administration [2]. For this reason, many materials for sustained delivery of VEGF have been studied [3, 4]. Microsphere-based drug delivery systems have been receiving increasing attention because they are injectable and are able to deliver multiple drugs [4–7].

Natural polymers such as collagen, gelatin, albumin, and hyaluronic acid have been used for drug delivery system (DDS) materials [8–11]. The advantage of employing such natural polymers is their excellent biocompatibility, compared to that of synthetic polymers. Among natural polymers, collagen exhibits superior biocompatibility because collagen is one of the major components of extracellular matrix and plays important roles in various biological events. Despite the advantages of collagen, little effort has been made to fabricate injectable collagen microspheres for VEGF delivery.

In this study, we prepared the injectable collagen microspheres for sustained release of VEGF using a water-in-oil emulsion system. First, the effects of surfactant concentration and rotating speed in emulsified mixture on the particle diameter were studied. Second, the microscopic observation and surface charge of the collagen microspheres were investigated. Third, we evaluated the release profile of recombinant human VEGF (rhVEGF) from the collagen microspheres. Finally, the bioactivity of rhVEGF released from the collagen microspheres were evaluated using a capillary formation assay of human umbilical vein

endothelial cells (HUVECs). Here, we demonstrate the method to control the particle diameter of the collagen microspheres in the range of 1–30 μm and characterize the collagen microspheres as a potential DDS material.

2 Materials and methods

2.1 Reagents

Native collagen from porcine skin was purchased from Nippon Meat Packers (Japan). Liquid paraffin, sorbitan monolaurate (Span 20), water-soluble carbodiimide (WSC), rhVEGF, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from WAKO Pure Chemical Industries (Japan). A VEGF ELISA kit and an antihuman CD31 immunostaining kit were purchased from invitrogen (Japan) and Kurabo (Japan), respectively. HUVECs, human normal dermal fibroblasts (NHDFs), and EGM-2 (growth medium for HUVECs) were purchased from Sanko Jyunyaku (Japan). EGM-2 was supplemented with human epidermal growth factor, hydrocortisone, FBS (2%), VEGF, basic fibroblast growth factor (bFGF), long R insulin-like growth factor-1 (IGF-1), ascorbic acid (Asc), gentamicin sulfate and heparin, as described by the manufacturer. EGM-2 without VEGF, bFGF, IGF-1, Asc, and heparin were prepared for a non-angiogenic medium (EGM) for HUVECs.

2.2 Preparation of collagen microspheres

Ten ml of 1% (w/v) collagen solution was emulsified in 50 ml of liquid paraffin containing surfactant at various concentrations (0–1.5% [v/v]) and stirred using a high power mixer (P-2, AsOne, Japan) at various rotating speeds (300–1,200 rpm) at room temperature for 5 min. For crosslinking, 1 ml of 50% (v/v) WSC in water was added to the emulsified mixture and kept stirring for 1 h to form collagen microspheres. Then, 50 ml of 50% (v/v) ethanol was added into the mixture and mixed for 5 min to separate the collagen microspheres from oil phase. The mixture was centrifuged at 3,500 rpm for 5 min and supernatant was removed. Ethanol (50% v/v) was added to the remained collagen pellet and mixed and centrifuged again (3,500 rpm, 5 min). After removal of supernatant, phosphate buffered-saline (PBS) was added to the collagen pellet and mixed and centrifuged (3,500 rpm, 5 min). This procedure was repeated 3 times to remove residual ethanol. The collagen microspheres for the experiments of VEGF impregnation, zeta potential, microscopic observation, in vitro release assay, and capillary formation assay were prepared under the conditions of the surfactant concentration of 0.3% and the rotating speed of 600 rpm.

2.3 VEGF impregnation in collagen microspheres

Impregnation of rhVEGF in the collagen microspheres was performed by immersing the collagen microspheres in the 1 μg/ml rhVEGF solution in PBS for 24 h at 4°C. The microspheres loaded rhVEGF were centrifuged at 3,500 rpm for 5 min and supernatant was removed. PBS was added to the collagen pellet and mixed and centrifuged (3,500 rpm, 5 min). This procedure was repeated 3 times to remove residual rhVEGF.

2.4 Particle size

The particle size of the collagen microspheres were measured by hand count. The collagen microspheres were stirred in PBS overnight to disperse homogenously. The pictures of the collagen microspheres were taken through a microscope (DMI6000B, Leica, Japan) and the diameters of the microspheres of at least 500 microspheres were measured by hand count.

2.5 Viscosity

The viscosities of liquid paraffin and collagen solutions (0.5, 1, and 1.5% [w/v]) at various temperatures were measured using a viscometer (viscostick; Shiro Industry, Japan).

2.6 Zeta potential

The surface charge of the collagen microspheres was determined by measuring the electrophoretic mobility using a Zetasizer (Nano ZS; Sysmex, Japan). The measurements were performed in PBS and EGM to investigate the solvents dependency of the zeta potential. A Smoluchowsky model was used to calculate the zeta potential values from the electrophoretic mobility. All measurements were carried out at 25°C in triplicate.

2.7 Microscopic observation

The collagen microspheres were fixed by 2.5% glutaraldehyde and were dehydrated by ethanol and subsequent isoamyl acetate. The samples were then freeze-dried and coated with Pt using an ion coater (L350S-C; Anelva, Japan) and subjected to SEM observation. The SEM apparatus (VE-9800; Kyence, Japan) was operated at 20 kV.

2.8 SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by using an XV pantera system (DRC, Japan). The concentration of separation gel was 5%.

The collagen microspheres were mixed in diluted HCl (pH 3) to a final concentration of 10 mg/ml. Collagen and gelatin for controls were diluted in diluted HCl (pH 3) to a final concentration of 0.1% (w/v) and 1% (w/v), respectively. The samples were mixed with 0.1 M Tris-HCl buffer (pH 6.8) containing 69.3 mM SDS, 40% (v/v) glycerol, 0.7 mM bromophenol blue, and 10% (v/v) 2-mercaptoethanol at the ratio of 1: 1 (v/v), heat at 99°C for 5 min, and then 10 µl was loaded per well of the XV pantera gel. After electrophoresis, gels were stained using 0.1% (w/v) Coomassie Brilliant Blue R250.

2.9 In vitro release study

The rhVEGF-loaded collagen microspheres (100 mg) were placed into insert wells (IntercellTP, Kurabo, Japan) and 400 µl of solvents were added into lower wells of a 24-well plate (Asahi Techno Glass, Japan). As the solvents, PBS, collagenase solution in PBS (1 U/ml), and EGM were used. The collagen samples were incubated at a constant temperature of 37°C. After specific time periods, the solvents were collected and replaced with fresh solvents and incubated again. The content of rhVEGF released in the collected solvents was determined by a VEGF ELISA kit according to the manufacturer's instruction. To assess the degradability of the collagen microspheres in collagenase solution (1 U/ml) and PBS, protein content measurement was performed using a bicinchoninic acid protein assay kit as previously described [12].

2.10 Capillary formation assay

Capillary formation of the HUVECs co-cultured on NHDFs was assessed according to the method previously described [13]. Conditioned EGM containing released rhVEGF were prepared as follows; EGM were incubated with the rhVEGF-loaded collagen microspheres at a constant temperature of 37°C for a week and collected, followed by replacement with fresh EGM and incubation for another week. This procedure was repeated 3 times for 4 weeks. The HUVECs co-cultured with NHDFs were treated with the conditioned EGM at 37°C in air containing 5% CO_2. As controls, fresh EGM and an EGM containing 2 nM (76.4 ng/ml) rhVEGF were used. At day 3 after the treatment, the cells were fixed and stained using an anti-human CD31 immunostaining kit according to the manufacturer's instruction. Stained area was calculated using an angiogenesis image analyzer (Kurabo).

2.11 Statistical analysis

Statistical analysis was performed using student's t tests, and a confidence level of 95% ($P < 0.05$) was considered necessary for statistical significance. The error bars indicate the standard deviation of the mean.

3 Results and discussion

3.1 Effect of surfactant concentration on particle size

Figure 1 shows the effect of surfactant concentration on the particle diameters. The average diameter, standard deviation, and variation coefficient were summarized in Table 1. The diameters proportionally decreased with increasing the surfactant concentration in the range of 0.1–0.5%. In this range, the diameters decreased at the ratio of 3.1 µm per 0.1% increase of surfactant concentration. There was little change in diameter at concentrations of more than 0.5%. Meanwhile, absent surfactant resulted in increased particle diameter and standard deviation. Student's t tests showed that the surfactant concentration significantly influenced the particle size except the difference between 1 and 1.5%. Considering that removal of the surfactant from the microspheres was more complicated with increasing surfactant concentration and that the variation coefficient of the condition of 0.3% was the lowest, the optimal surfactant concentration was decided to be 0.3%.

3.2 Effect of rotating speed on particle size

Figure 2 shows the effect of rotating speeds on the particle diameters under the condition of 0.3% surfactant concentration. The average diameter, standard deviation, and variation coefficient were summarized in Table 2. The diameters proportionally decreased with increasing the rotating speed at the ratio of 1.8 µm per 100 rpm increase of the rotating speed, although the variation coefficient was the lowest at the rotating speed of 600 rpm. Student's t tests showed that the rotating speed significantly influenced the particle size. Consequently, the particle diameter could be controlled by changing the rotating speed and the rotating speed of 600 rpm was needed to make finer microspheres.

3.3 Effects of viscosity and temperature on particle size

Viscosity and reaction temperature of the emulsified mixture were important factors to control the particle size of the collagen microspheres. The viscosity of oil phase decreased with increasing temperature, although that of water phase showed little change (Fig. 3a). In addition, increased viscosity of oil phase resulted in decreased size of the particle diameter (Fig. 3b). It can be speculated that decreased viscosity of oil phase

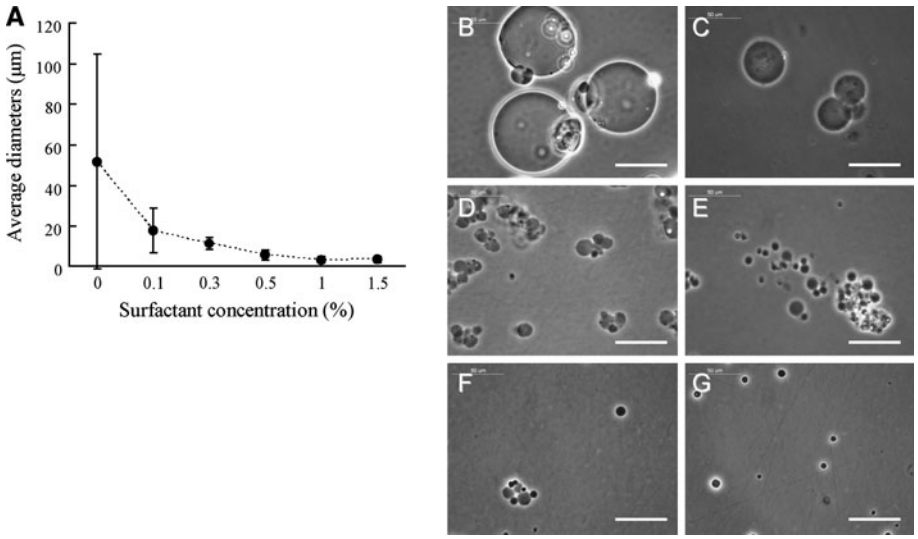

Fig. 1 Effect of surfactant concentrations on particle diameters (**a**). Photographs show the collagen microspheres prepared under the surfactant concentrations of 0% (**b**), 0.1% (**c**), 0.3% (**d**), 0.5% (**e**), 1% (**f**), and 1.5% (**g**), respectively. Rotating speed was set to 600 rpm. Bars: 50 μm

Table 1 Effect of surfactant concentrations on diameter and dispersity of collagen microspheres

Surfactant concentration (%)	Average diameter (μm)	Standard deviation (μm)	Variation coefficient	Size range (μm)
0	51.68	53.14	1.028	5.26–315.7
0.1	17.77	11.27	0.634	2.65–71.43
0.3	11.38	3.16	0.278	3.36–21.01
0.5	5.49	2.58	0.470	1.68–16.81
1.0	3.21	1.63	0.508	0.84–12.61
1.5	3.36	1.53	0.455	0.42–10.92

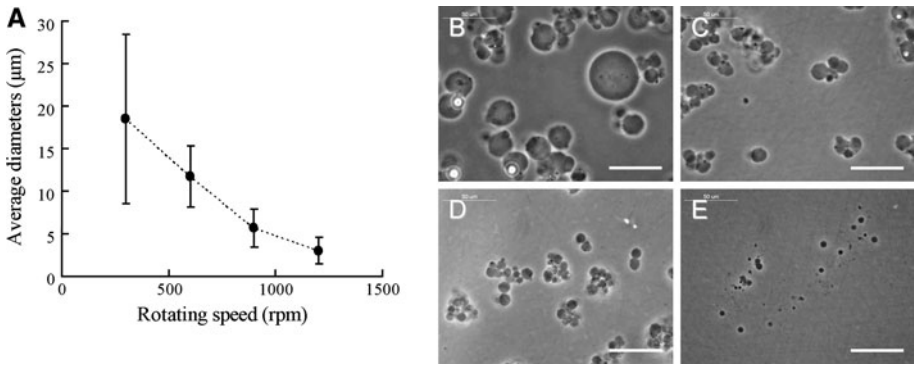

Fig. 2 Effect of rotating speeds in emulsion system on particle diameters (**a**). Photographs show the collagen microspheres prepared under the rotating speeds of 300 rpm (**b**), 600 rpm (**c**), 900 rpm (**d**), and 1,200 rpm (**e**), respectively. Surfactant concentration was set to 0.3%. Bars: 50 μm

Table 2 Effect of rotating speeds on diameter and dispersity of collagen microspheres

Rotating speed (rpm)	Average diameter (μm)	Standard deviation (μm)	Variation coefficient	Size range (μm)
300	18.51	9.92	0.536	2.63–92.11
600	11.73	3.60	0.307	2.52–25.21
900	5.71	2.25	0.394	0.84–11.76
1200	3.01	1.54	0.512	0.42–9.24

resulted in the formation of finer emulsion and larger droplets. On the other hand, increased collagen concentration of water phase also altered the particle diameters (Fig. 3c). The viscosity of collagen solution increased with increasing the collagen concentration (Fig. 3a), therefore, the increased viscosity in water phase resulted in the increase of the particle diameter. Consequently, viscosity and temperature of the emulsified mixture

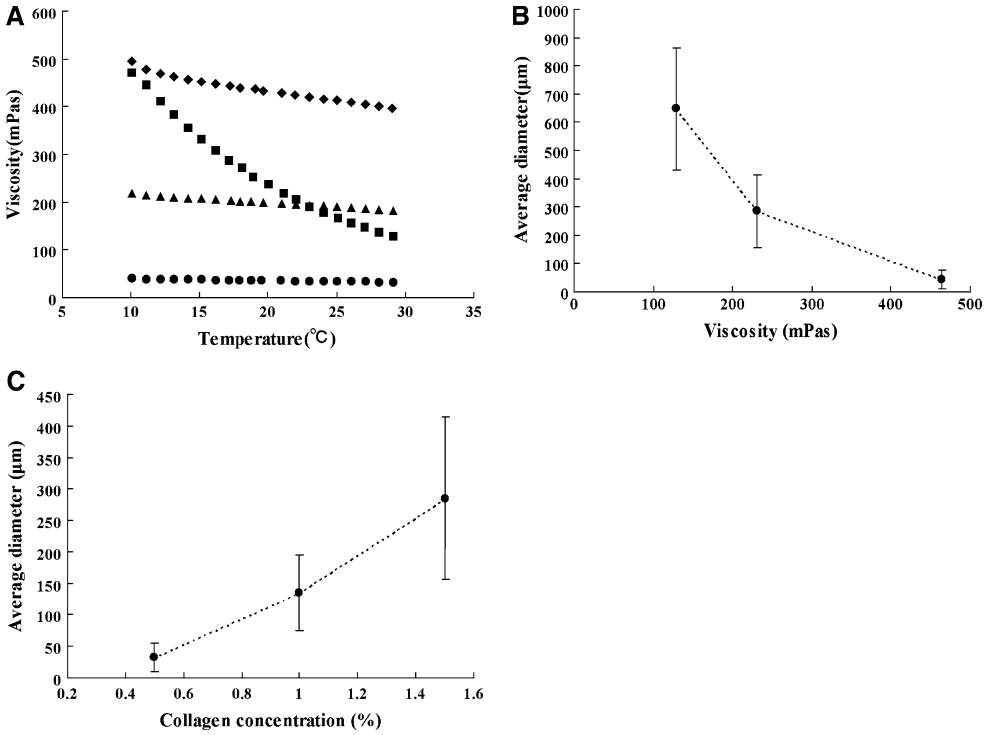

Fig. 3 **a** Effect of reaction temperature on the viscosities of liquid paraffin (*squares*), 0.5% collagen (*circles*), 1.0% collagen (*triangles*), and 1.5% collagen solutions (*diamonds*), respectively. **b** Effect of viscosity in oil-phase on particle diameters. **c** Effect of collagen concentration in water-phase on particle diameters. Surfactant concentration and rotating speed were set to 0.1% and 500 rpm, respectively

should be controlled to make the collagen microspheres having intended particle diameters.

3.4 Zeta potential

The surface charge of the collagen microspheres was determined by measuring their electrophoretic mobility. Table 3 shows the zeta potential values of the collagen microspheres in PBS and EGM. The collagen microspheres in the present study had slight positive charge. Berthold et al. [8] have reported that marine collagen microspheres showed negative charge of about −30 mV at pH 7. The difference could be caused by the types of crosslinking agents used. The present study used WSC, which forms crosslinks between amino groups and carboxyl groups by condensation reaction [14]. On the other hand, previous reports used glutaraldehyde, which forms crosslinks between only amino groups and has some incomplete reaction of the bifunctional reagent leading to free aldehyde groups [8]. Therefore, the charge distribution of the functional groups in collagen molecules might cause the difference in the zeta potential compared to the previous studies. Meanwhile, the zeta potential in EGM was slightly lower than that in PBS. It has been reported that the electrostatic binding of buffer anions to the surface of the positively charged microspheres could be observed [15, 16]. Therefore, the difference in the concentration of buffer anions and ionic strength between PBS and EGM might influence the zeta potential.

3.5 SEM and SDS-PAGE

SEM photographs show spherical particles with a smooth surface and a diameter of ca. 2.5 μm (Fig. 4). Some of the particles were found to be aggregated (data now shown). Low zeta potential of the collagen microspheres could lead the aggregation due to the interparticle attraction forces such as van der Waals' force. We performed the SDS-PAGE analysis to prove that the microspheres were indeed collagen and not denatured, but could not see any bands of collagen as well as those of gelatin (data not shown), indicating that the chemical crosslinks between collagen molecules made it difficult to denature the collagen microspheres by heat. Our previous experiments have demonstrated that the collagen sponge crosslinked with WSC had over 100°C of denaturation peak in differential scanning calorimeter analysis (unpublished data), which may explain the SDS-PAGE results. Raman spectroscopy and Fourier transform infrared spectroscopy are needed to prove the collagen denaturation in future works.

Table 3 Zeta potential values of collagen microspheres in PBS and EGM

Solvents	Zeta potential (mV)
PBS	8.86 ± 0.30
EGM	3.15 ± 0.63

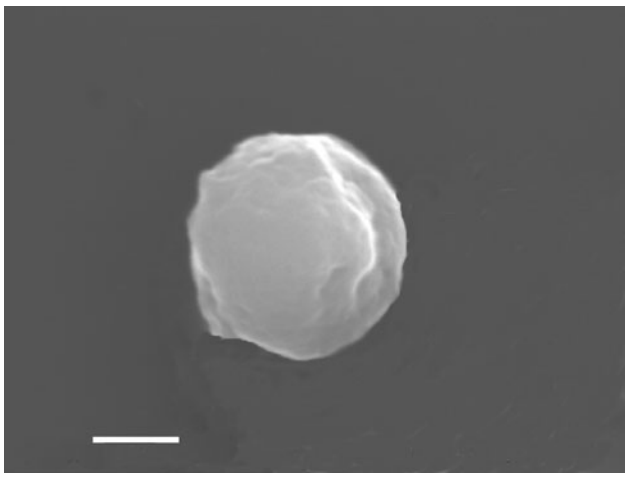

Fig. 4 SEM photograph of collagen microspheres. The microspheres were prepared under conditions of rotating speed of 600 rpm and surfactant concentration of 0.3%. Magnification: ×15,000. Bar: 1 μm

3.6 Release profile of rhVEGF

The release profile of rhVEGF from the collagen microspheres was different among the solvents used (Fig. 5a). In the case of collagenase solution, the rhVEGF released with a rapid rate during initial 8 days, followed by a slower release rate for the remaining duration of the experiment.

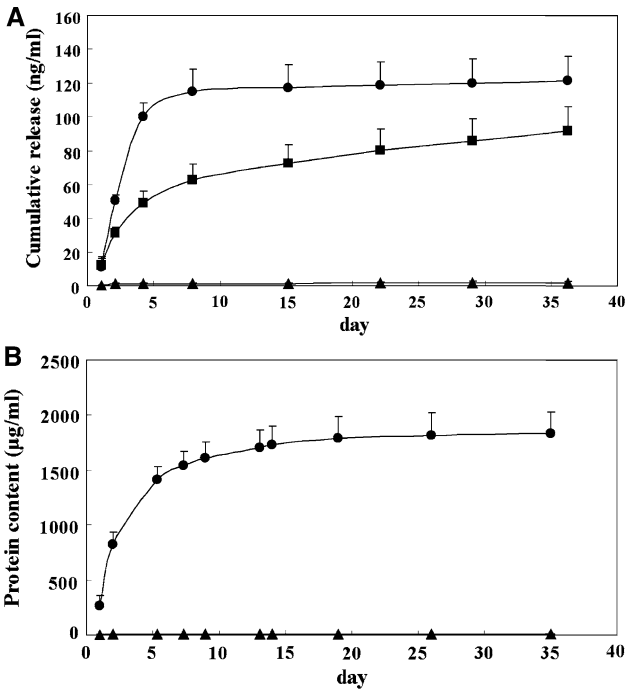

Fig. 5 **a** In vitro release profile of rhVEGF-loaded collagen microspheres in collagenase solution (*circles*), PBS (*triangles*), and EGM (*squares*). **b** In vitro degradation rate of collagen microspheres in collagenase solution (*circles*) and PBS (*triangles*)

The release curve in collagenase solution was similar to the degradation curve of the collagen microspheres in collagenase solution (Fig. 5b), indicating that the drug release could occur by degradation of the collagen microspheres. The collagenase concentration (1 U/ml) used in this study was in the physiologically relevant concentration range [17], therefore, the collagen microspheres might show the same release profile as the collagenase solution results. Meanwhile, the release rate into EGM was higher than that into PBS, indicating that drug diffusion between EGM and PBS could be occurred. Additionally, the difference in the charge state of the collagen microspheres in the solvents might affect the release rate (Table 3). It is believed that there are binding forces, such as electrostatic and hydrophobic interactions, between collagen and protein [18]. Therefore, the change in the electrostatic interaction between the collagen microspheres and rhVEGF might be one of the reasons for the difference in the release profile. Consequently, rhVEGF could be released by drug diffusion as well as collagen degradation.

3.7 Bioactivity of released rhVEGF

An in vitro capillary formation assay was performed to evaluate whether the released rhVEGF remained bioactive (Fig. 6). We have reported that the HUVECs co-cultured on NHDFs showed capillary-like structures in the presence of VEGF in the EGM [13]. The HUVECs co-cultured on NHDFs were exposed to the conditioned EGM which were collected after incubation with rhVEGF-loaded collagen microspheres for 0–7, 8–14, 15–21, and 22–28 days. Cells treated with the conditioned EGM showed capillary formation although the total length of the capillary was small compared to the control from the area calculation results using a software analysis (data not shown) and became smaller with increasing incubation periods. This can be explained from the release profile results of EGM (Fig. 4a, squares); the release amount of rhVEGF in EGM became lower with increasing incubation periods. The total amounts of rhVEGF released at 0–7, 8–14, 15–21, and 22–28 days were 56.5, 15.0, 8.8, and 6.2 ng/ml, respectively, which were lower than the control (76.4 ng/ml). Consequently, the bioactivity of rhVEGF was maintained after release as observed capillary formation.

There are some reports to make collagen microspheres for tissue engineering. Kim et al. [6] reported the collagen-apatite composite microspheres for bone tissue engineering with average diameter of 166 μm. Swatschek et al. [19] reported the agglomated collagen microspheres for retinol delivery with average diameter of 2.2 μm. Berthold et al. [8] reported the collagen microspheres for glucocorticosteroids delivery with average diameter of 10 μm. Recently, Chan et al. [20] reported the nano-

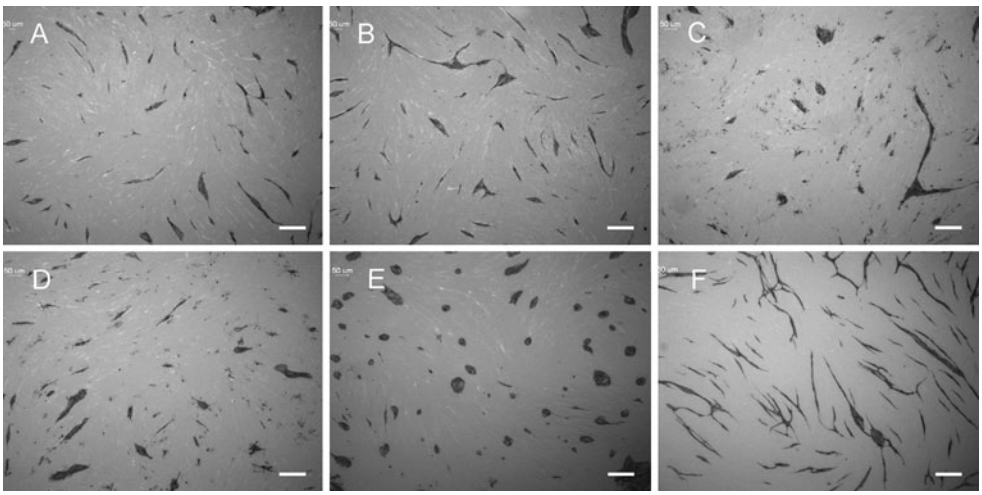

Fig. 6 Capillary forming ability of HUVECs cultured in conditioned EGM which were collected after incubation with rhVEGF-loaded collagen microspheres for 0–7 days (**a**), 8–14 days (**b**), 15–21 days (**c**), and 22–28 days (**d**). Controls were untreated cells (**e**) and the cells treated with EGM containing 2 nM rhVEGF (**f**), respectively. Bars: 100 μm

fibrous collagen microspheres with average diameter of 70 μm and demonstrated their release profiles of nerve growth factor. The present study demonstrated smaller collagen microspheres having 1–30 μm diameters and the release profile of bioactive rhVEGF during 4 weeks incubation. Additionally, the present study first provides the technique for easy control of particle diameter of collagen microspheres. Crosslinker WSC used in this study is known to be biocompatible because WSC do not persist in the linkage between collagen molecules and are simply washed away after crosslinking [21]. Consequently, the collagen microspheres have potential use for clinical applications, such as injectable DDS biomaterials for tissue engineering.

4 Conclusions

We successfully fabricated the collagen microspheres for sustained release of rhVEGF. The particle size can be easily controlled by changing surfactant concentrations and rotating speeds in the process of emulsification. The sustained release of rhVEGF was achieved for 4 weeks and the released rhVEGF remained bioactive. This work contributes to future development of collagen microsphere-based protein delivery system for tissue engineering.

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