J Mater Sci: Mater Med (2009) 20:1299–1305
DOI 10.1007/s10856-008-3681-1

# Preparation and characterization of gelatin sponge millispheres from air-in-water-in-oil-type emulsions

**Noboru Yamashita · Satoshi Izumikawa · Akira Takagi · Haruyuki Arakawa · Tatsuyoshi Wakasawa · Atsushi Maruyama**

Received: 11 August 2008 / Accepted: 22 December 2008 / Published online: 10 January 2009


**Abstract** A novel method for the preparation of gelatin sponge millispheres (GSMs) for biomaterials such as embolic agents and cell scaffolds was developed using an air-in-water-in-oil-type emulsion. The droplets, consisting of a foamy gelatin suspension in caprylic triglyceride, were gelled and rinsed with isopropanol. Sonication and depressurization were used during the rinsing process to create interconnected pores. GSMs cross-links created over 4 h at 155°C without any agent were insoluble and had short and long diameters of $1.1 \pm 0.2$ mm and $1.3 \pm 0.2$ mm, respectively. The residual isopropanol and caprylic triglyceride were <0.05% (w/w) and <1% (w/w) respectively. The level of bacterial endotoxins in the extracts was below 0.025 EU/ml, and no bacterial or fungal growth was found during sterility testing. The GSMs produced using this method were considered to meet the basic requirements of embolic agents.

## 1 Introduction

Estimates based on data obtained from 2000 to 2004 indicate that liver cancer remains the fifth most common malignancy in men and the eighth in women worldwide. The incidence of primary liver cancer is increasing in several developed countries, including the United States, and this increase is likely to continue for the next decade [1]. Curative therapies for hepatocellular carcinoma (HCC), such as resection, liver transplantation, or percutaneous ablation, can be applied in selected patients with early tumors, which account for approximately 30–40% of all clinical cases. In more advanced stages, however, systemic or local methods like therapeutic embolization and transcatheter arterial embolization [TAE, also known as transcatheter arterial chemoembolization (TACE)] are necessary. Treatment of HCC with TAE was first reported by Doyon et al. [2], after which it was intensively developed as transcatheter arterial chemoembolization (TACE) in Japan by Yamada et al. [3]. TACE, a technique combining intra-arterial chemotherapy and selected ischemia, has shown a modest survival advantage in two randomized controlled trials and a meta-analysis, and is currently the mainstay of treatment for these stages [4].

Two types of embolic agents have been used for HCC TACE. One type is permanent, such as tris-acryl gelatin microspheres [e.g. Embosphere® (BioSphere Medical, Inc., USA)] [5], and the other is temporary, such as gelatin sponge fragments (Gelfoam®, Pfizer Inc., USA and Spongel®, Astellas Pharm Inc., Japan) [6–8]. One advantage of temporary embolization is recanalization, which can be useful for repeated embolizations or when there is no concern about the long-term effects of the treatment [7, 8]. Another advantage is that gelatin sponge fragments are more flexible than microspheres, which makes them more catheter-compatible [9]. In addition, their spongy character might also be useful in absorbing contrast medium and anticancer agents, which are delivered to the target site through the catheter along with the sponge fragments [3].

N. Yamashita (✉) · S. Izumikawa · A. Takagi · H. Arakawa · T. Wakasawa
Pharmaceutical Research and Technology Labs., Institute for Technology, Astellas Pharma Inc., 180 Ozumi, Yaizu-shi, Shizuoka-ken 425-0072, Japan
e-mail: noboru.yamashita@jp.astellas.com

A. Maruyama
Institute for Materials Chemistry and Engineering, Kyushu University, 744-CE11 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

Although fragments made by hand from a sheet of gelatin sponge using surgical tools (e.g. a surgical knife) have frequently been used for temporary embolization, in accordance with the particular indications of HCC [3], the preparation of the fragments is troublesome and carries a risk of exogenous bacterial infection of the liver [10]. Furthermore, this use of gelatin sponge for embolization is off-label in the USA, Japan, and European countries. Despite these disadvantages, gelatin remains a popular choice because it is a biodegradable material, and thus presents no concern of long-term effects on the body.

Spherical agents (e.g. tris-acryl gelatin microspheres) are reportedly preferable to those with irregular shapes (e.g. PVA foam) because occlusions do not occur at undesirable sites, such as in the catheter [9]. Many clinicians have long sought such spherical gelatin sponge products with a diameter of approximately 1 mm for use as HCC embolic agents [11]. However, it is difficult to carve spheres of a specific size out of a gelatin sponge sheet by hand under sterile conditions. In recent years, the gelatin sponge GelitaSpon IR GS-322 (Gelita Medical B.V., Amsterdam, The Netherlands) has been commercially available in Europe; however, this product is cubic and larger ($3 \times 2 \times 2$ mm) than that in clinical demand for use as an HCC embolic agent. Although the use of porous gelatin microspheres under 500 μm in diameter as scaffolds for anchorage-dependent cells has been reported [12], they were manufactured using conventional o/w/o type emulsification with a potentially harmful solvent (toluene), detergent/emulsifier (Tween 80/Span 85), and chemical cross-linker (glutardialdehyde) [13]. These microspheres, then, might not be suitable for use as embolic agents for HCC because of their small size and potential toxicity.

In this study, a novel preparation method for gelatin sponge millispheres (GSMs) was developed to provide a reliable and convenient supply of embolic agents for TAE. GSMs were characterized and preliminarily tested for their biocompatibility.

## 2 Materials and methods

### 2.1 Materials

Special grade gelatins meeting the specifications of the Japanese Pharmacopeia XIV (JP) were kindly provided by Jellice Co. (Miyagi, Japan) and Nitta Gelatin Co. (Osaka, Japan). Panacete 800 (caprylic triglyceride) was purchased from NOF Corporation (Tokyo, Japan). Special grade isopropanol meeting the specifications of the Japanese Pharmacopeia XIV (JP) was purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). All liquid materials for GSM preparation were sterilized by filtration (0.22 μm) before use.

### 2.2 Preparation of GSMs

A flow chart of the GSM preparation procedure is shown in Fig. 1. Gelatin was dissolved in water for injection (WFI) in a stainless steel vat at a concentration of 4% (w/v), with agitation and warming in a water bath. Following sterilization by filtration (0.22 μm), the gelatin solution was vigorously agitated with a paddle stirrer (Mazela Z, Tokyo Rikakikai, Japan) to form an aerated foam. The foamy gelatin solution was poured into a vat containing caprylic triglyceride and agitated to form an air-in-water-in-oil-type emulsion. The size of the droplets in the suspension was regulated by the speed of the agitation. The vat was then cooled in a chilled water bath to generate gelled particles from the aqueous droplets through the temperature-dependent sol/gel phase transition of the gelatin. These gelled particles were first dehydrated by rinsing with chilled isopropanol, after which they were further rinsed under sonication and depressurization to facilitate pore generation in and on the particles. Rinsed particles of around 1 mm were selected by sieving them through

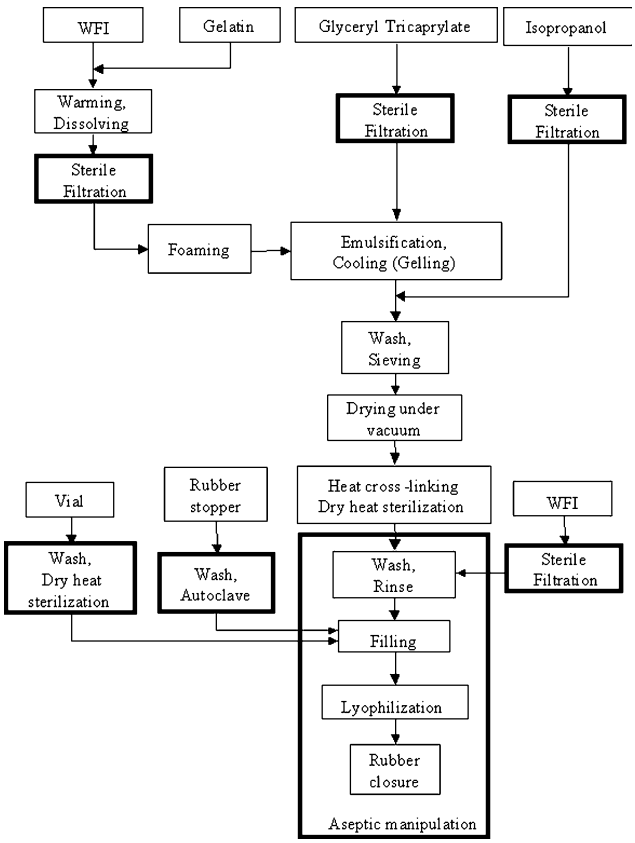


**Fig. 1** Preparation procedure for gelatin sponge millispheres (GSMs)

punched metal containers while agitating in chilled isopropanol. The recovered particles were heat-treated in a vacuum drying oven (VO-320, Advantec, Japan) at various temperatures (135–200°C) for various time periods [0.5–24 h] to cross-link the gelatin. The temperature and time period for each condition were variations of the established conditions (155°C, 4 h) for the gelatin sponge sheet (unpublished data). The cross-linked particles were then dispersed in water to remove the soluble component, and the resulting particles were dispensed in vials, lyophilized under aseptic manipulation, and stored at 5°C or at room temperature until use. Samples prepared without foaming with agitation or without rinsing with sonication and depressurization were prepared as control samples. Cubic fragments of gelatin sponge sheet (Spongel®, Astellas, Japan) were also prepared as control samples by hand using a razor.

## 2.3 Characterization of GSMs

### *2.3.1 Insolubility*

One hundred milliliters of dried particles were dispersed in 1 l of Milli-Q water. After agitation for 5 min, the excess water in the dispersion was filtered through a G1-sintered glass funnel. The insolubility of the particles was evaluated by visual inspection and ranked into four grades as follows: "soluble," residual particles were not observed; "partially insoluble," residual particles were dissolved or swollen with hardly any particles of the initial size remaining; "insoluble," residual particles of the initial size were observed; and "insoluble but discolored," changes in the color of insoluble particles were observed upon visual inspection.

### *2.3.2 Lysine residues*

Lysine residues in the GSMs and the raw material gelatin were determined using a modification of a previously established method [14]. Briefly, a coloring reaction between trinitirobenzene sulfonic acid (TNBS) and the $\varepsilon$-amino groups of the lysine residues was employed. The unmodified lysine residue content in the GSMs and original gelatin were calculated according to a previous report [14], and expressed as moles lys/g gelatin.

### *2.3.3 Scanning electron microscopy*

GSM specimens were fixed on specimen mounts with adhesive tape, and coated with Pt using an auto fine coater (JFC-1600, JEOL, Japan). Morphology was observed using a scanning electron microscope (JSM-5510LV, JEOL, Japan).

### *2.3.4 Particle size*

The particle sizes of GSMs were evaluated by light microscopic observation. Eighty milligrams of GSMs were dispersed in 30 ml of saline. A portion of each sample dispersion so obtained was taken for particle size determination. Long and short diameters of 20 randomly extracted particles ($n = 3$) from each sample (3 batches) were measured.

### *2.3.5 Residual isopropanol*

Accurately weighed 0.05 g aliquots of the specimen were dissolved in 5 ml of 1 N NaOH. Two and half milliliters of an internal standard (1-propanol diluted in 1 N HCl at a dilution ratio of 3:40,000) was added to 2.5 ml aliquots of the solution, followed by dilution with Milli-Q water to give a final volume of 50 ml. Fresh samples were prepared at each examination. The concentration of isopropanol in each sample solution was determined by gas chromatography (HP6890, Hewlett Packard, USA).

### *2.3.6 Residual caprylic triglyceride*

Accurately weighed 0.1 g aliquots of the specimen were dissolved in 5 ml of potassium hydroxide ethanol solution and refluxed for 30 min. After cooling, 8 ml of 1 N HCl was added. The solution was extracted twice with 12 ml hexane. The hexane phase was collected and evaporated. The evaporated residual was dissolved in 2 ml of mobile phase [a mixture of 0.05 M $NaH_2PO_4$ (pH 3) and acetonitrile (6:4)] for HPLC analysis. Calibration samples were freshly prepared for each examination. The concentration of caprylic acid produced from the caprylic triglyceride in the sample solutions was determined using HPLC (LC-10A, Shimadzu, Japan) using a UV detector (210 nm).

## 2.4 Microbiological properties of GSMs

### *2.4.1 Bacterial endotoxins*

Eighty milligrams of GSMs were suspended in 40 ml of Limulus amoebocyte lysate (LAL) reagent with agitation and left at room temperature for 1 h. Calibration samples were freshly prepared for each examination. The concentrations of bacterial endotoxins in the supernatant of the GSM suspension were determined using the turbidimetric method for endotoxin testing as described in the JP.

### *2.4.2 Sterility*

Since GSMs are insoluble, a sterility test was conducted according to the Direct Inoculation Method, as stipulated

under the Sterility Test section of the General Test Methods described in the JP.

## 3 Results and discussion

### 3.1 Insolubilization of millispheres by heat cross-linking

The insolubility of therapeutic embolization devices is the most important property required for the embolization of arteries in which blood still flows. Because gelatin is generally soluble in aqueous solution (e.g. imaging agent or blood), it needs to be insolubilized before it can be used for embolization. From a toxicity point of view, heat (as opposed to chemical) cross-linking is considered to be the most favorable method for accomplishing this. Table 1 summarizes the insolubility of GSMs after heat treatment under various conditions. Insolubilization seems to be time- and temperature-dependent, as particles treated at 135°C for 6 h remain soluble, while those treated at temperatures over 190°C for 1 h are insoluble (Table 1). However, GSMs treated at temperatures over 190°C underwent a significant color change (i.e. from white to black or dark brown). This was considered to be due to the carbonization of the gelatin, which may result in a depletion of elasticity. It was therefore concluded that the best results were obtained with 4 h of treatment at a temperature between 155 and 165°C. These conditions are consistent with those for gelatin sponge sheets (also treated at 155°C for 4 h), which are conventionally used in clinical cases.

The number of lysine residues with unmodified ε-amino groups was found to characterize the chemical properties of GSMs. The number of primary amino groups in the GSMs was 14.4 ± 3% ($n = 3$) less than that in the raw material gelatin [$3.27 \pm 0.2 \times 10^{-4}$ mol/g ($n = 6$)]. The decrease is thought to correlate with the degree of gelatin cross-linking, based on commonly proposed mechanisms [15]. One possible mechanism might involve the formation of desmosine-type cross-linking, which is known to occur in elastin fiber [16]. Lysine residues, which are proximal to each other, are oxidatively deaminated to yield terminal aldehyde groups. One of the aldehyde groups could then be attacked by the free ε-amino group of a neighboring lysine to yield an imine, which would subsequently undergo a series of aldol-type condensation reactions to produce a cross-linked product containing pyridinium ring(s). The decrease in the amount of lysine in the GSMs was thought to be correlated to the degree of gelatin cross-linking.

**Table 1** Effect of various heat cross-linking conditions during the insolubilization of GSMs

| Temperature (°C) | Heating time (h) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0.5 | 1 | 2 | 3 | 4 | 6 | 24 |
| 135 | – | – | – | S | S | S | – |
| 155 | – | – | PI | – | I | I | – |
| 160 | – | – | PI | I | I | – | – |
| 165 | – | – | PI | PI | I | – | – |
| 170 | – | – | I | – | – | – | – |
| 180 | S | PI | – | – | – | – | – |
| 190 | PI | IC | – | – | – | – | – |
| 200 | S | IC | – | – | – | – | IC |

Scale of insolubility for GSMs ($n = 1$)

*S* soluble, *PI* partially insoluble, – not determined, *I* insoluble, *IC* insoluble but discolored

### 3.2 Formation of millispheres with interconnected pores

The minute structure of the GSMs was observed with a scanning electron microscope. The GSMs were spherical (Fig. 2a), and, like the gelatin sponge sheet fragments (Fig. 2c), their surfaces and interiors were porous (Fig. 2b). Importantly, these pores were interconnected. This structural pattern was thought to be generated during the washing and rinsing step, when depressurization and sonication is employed, since the pores in particles that weren't depressurized and sonicated were closed (Fig. 2d). These interconnecting pores permit the effective inflow of external fluids and outflow of air, which allows the GSMs to be effectively washed to remove impurities. This feature is highly important in avoiding the retention of air, water, or caprylic triglyceride within the particles due to insufficient rinsing. Residual water could cause particles to aggregate since non-cross-linked gelatin could be dissolved by the water and acts as a binder (data not sown). Residual oily phase (i.e. caprylic triglyceride) could confer undesirable characteristics such as water-shedding, which would cause problems with the preparation of the dosing solution. Air encapsulated in the particles (e.g. Fig. 2d, e) could cause undesirable properties, such as buoyancy, which would also cause problems with the preparation of the dosing solution. In addition, if residual air is released after administration, it might cause an undesirable embolization, which could be fatal. Another benefit of particles made from gelatin foam is that the thin walls are useful for maintaining flexibility within the catheter. In contrast, the fleshy structure of particles prepared without the foaming step (Fig. 2e) might cause obstruction in the catheter.

The short and long diameters of GSMs were 1.1 ± 0.2 mm and 1.3 ± 0.2 mm respectively, yielding a short/long diameter ratio of 86%. The target size of GSMs were determined based on interviews with physicians at 19 major Japanese medical institutions that routinely perform

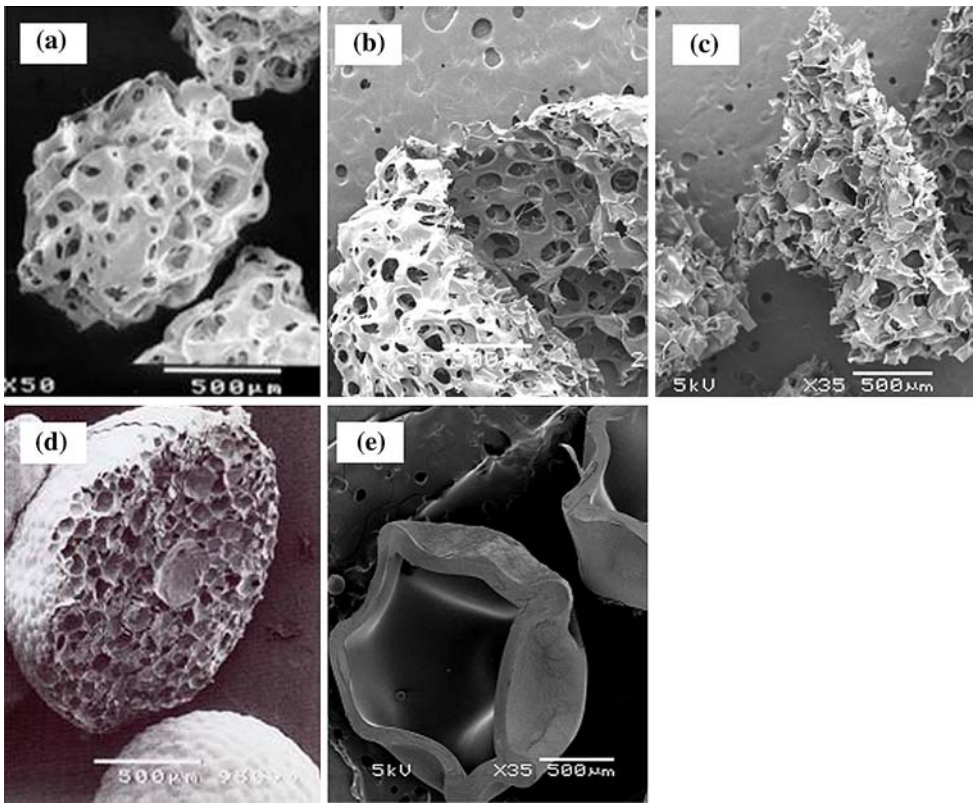


**Fig. 2** Representative scanning electron micrograph of gelatin sponge millispheres (GSMs), gelatin sponge fragments, and gelatin spheres: **a** surface of GSMs; **b** cross-section of GSMs; **c** gelatin sponge sheet fragments; **d** cross-section of GSMs rinsed without using depressurization or sonication; **e** cross-section of gelatin spheres prepared using the GSM method without the foaming step

therapeutic embolization against HCC using conventional gelatin sponge fragments. The target size of the fragments used in most institutions was approximately 1 mm (unpublished data). The size of GSMs was controlled mainly by regulating the speed of agitation during the emulsification of the gelatin foam in the oily phase (i.e. caprylic triglyceride). Particle size could be further regulated by sieving. GSMs with diameter of approximately 2 mm could also be prepared by using slower agitation and a sieve with larger pores (data not shown). Importantly, fatal pulmonary complications were seen after embolization with smaller sizes of tris-acryl gelatin-coated microspheres (40–120 µm) [5]. In addition, small foci of infarction in non-tumor areas of the resected liver, as well as gallbladder infarctions, were observed after embolization with gelatin sponge powder (<300 µm) [17] or (<500 µm) [18]. These effects were not observed with GSMs, which are thought to have a low risk of such adverse events.

### 3.3 Compliance with requirements for residual solvents in medical devices

The residual amount of isopropanol in the GSMs ($n = 3$) was below the detectable limit [0.05% (wt)]. Residual solvents in medicinal products are strictly regulated by the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use [19]). Isopropanol is categorized as a Class 3 solvent, which may be regarded as less toxic and of lower risk to human health. A residual amount of <0.5% of solvents of this grade is considered acceptable, and virtually all residual isopropanol in GSMs should be eliminated by heat cross-linking at 155°C (boiling point of isopropanol: 82°C). In addition, after heat cross-linking, GSMs can be rinsed with water, which is miscible with isopropanol. Rinsing with water followed by lyophilization under vacuum might also be an effective way to further reduce the amount of residual isopropanol in GSMs.

The residual amounts of caprylic triglyceride found in the GSMs are summarized in Table 2. Residual triglyceride of <1% (w/w) was detected in all GSM samples ($n = 3$) from three different batches. The residual caprylic triglyceride in GSMs decreased dramatically after creating interconnected pores via sonication and depressurization while rinsing with isopropanol (data not shown). Furthermore, it is impossible to rinse GSMs thoroughly without

**Table 2** Residual caprylic triglyceride in GSMs

| Batch | % Residual caprylic triglyceride (w/w) (Mean ± SD, $n = 3$) |
|---|---|
| 1 | 0.18 ± 0.05 |
| 2 | 0.12 ± 0.03 |
| 3 | 0.18 ± 0.03 |

the interconnected pores because they float in the rinsing solvent (isopropanol). Prior to this analysis, 84% recovery was achieved in the addition-recovery test performed on GSMs spiked with 0.05% (wt) of caprylic triglyceride (relative standard deviation: 4.3%). The toxicity of caprylic triglyceride has been reported to be very low as it is a normal body constituent found in fat cells and chylomicrons [20–22]. Fat emulsions consisting of mainly medium chain triglycerides (MCTs: caprylic and capric triglycerides) are used in humans for parenteral feeding (e.g. Lipofundin® MCT/LCT, B. Braun Melsungen AG, Germany) and as carrier for lipohilic compounds (e.g. propofol: Propofol-®Lipuro, B. Braun Melsungen AG, Germany) in Europe [23]. Therefore, it seems unlikely that residual amounts of caprylic triglyceride in GSMs would pose a problem in clinical practice.

### 3.4 Compliance with microbiological requirements for medical devices

Endotoxin concentrations in extracts from GSMs were below the detection limit (0.025 EU/ml). Endotoxins are of particular concern to those manufacturing medical devices as they are among the most potent pyrogens that can contaminate a product. Patients infected with endotoxin via the vascular or lymphatic routes can present with severe fever, shock, and even death [24]. Medical devices must meet varying levels of endotoxin control, depending on their clinical use; those that come in contact with cerebrospinal fluid and the circulatory system, including the lymphatic system, have the most stringent levels of endotoxin control. The US has established guidelines indicating the limits for acceptable numbers of endotoxin units (EU) in medical devices. The guidelines require that there be <0.5 EU/ml in water extracted from a device. The endotoxin content in gelatin was thought to be an important determinant of the amount of endotoxins in GSMs since the heat cross-linking process (155°C for 4 h) is not sufficient for depyrogenation. Temperatures over 180°C (normally 250°C, >30 min) are necessary to inactivate endotoxins [25]. Therefore, low-endotoxin gelatin (as determined by endotoxin assay) was selected as the raw material for GSMs (unpublished data).

No bacterial or fungal growth was detected during GSM sterility testing using the direct method. In a separate test performed prior to sterility testing, the GSMs were confirmed to be free of substances that inhibit microbial growth (data not shown). The GSMs were processed after sterilizing by heat cross-linking (155°C for 4 h) under aseptic conditions confirmed via the media-fill test. The sterility of GSMs is therefore unlikely to pose a problem in clinical practice.

In addition, GSMs are considered to be stable since no changes in the properties described above were detected after storage at room temperature for at least 3 years (data not shown).

Additionally, we consider that GSMs may be useful not only as therapeutic embolization devices, but also for fundamental applications in other biotechnology areas (e.g. tissue engineering), given that their unique properties (i.e. injectability, interconnected-pore structure, biocompatibility, and sterility) render them suitable as biomaterials (e.g. cell scaffold [12]).

## 4 Conclusion

GSMs were prepared via a novel method using air-in-water-in-oil emulsification, followed by sol–gel transition, dehydration with isopropanol, vacuum drying, and heat treatment for cross-linking. No harmful chemicals are needed in this method. GSMs are spherical particles with interconnected pores not only on the surface, but also on the interior proximal surfaces between air spaces. They are insoluble in water and disperse easily due to their spherical shape, which is widely recognized as the ideal for embolic agents. This is the first report of microspheres with these attributes made of commonly used gelatin sponge. The size of the GSMs can be maintained at around 1 mm, which is the same size as the labor-intensive cubic gelatin sponges manually prepared by clinicians.

The results of analytical and microbiological testing for impurities (caprylic triglyceride, isopropanol, endotoxins, bacteria, and fungus) indicated that GSMs have the general properties necessary for use as an HCC embolic agent. These properties may allow for not only the reliable and convenient supply of embolic agent, but also application to other biomedical fields (e.g. injectable scaffolds for tissue regeneration).

**Acknowledgements** The authors thank Dr. Ryusaku Yamada (MD), professor emeritus of Osaka City University, and Dr. Hironobu Nakamura (MD, PhD), professor of Osaka University Graduate School of Medicine, for coordinating our surveillance studies at major medical facilities in Japan.

## References

1. F.X. Bosch, J. Ribes, M. Diaz, R. Cleries, Gastroenterology **127**, S5 (2004). doi:10.1053/j.gastro.2004.09.011
2. D. Doyon, A. Mouzon, A.M. Jourde, C. Regensberg, C. Frileux, Annales de Radiol. **17**, 593 (1974)
3. R. Yamada, K. Kishi, T. Sonomura, M. Tsuda, S. Nomura, M. Satoh, Cardiovasc. Intervent. Radiol. **13**, 135 (1990). doi:10.1007/BF02575464
4. J. Bruix, M. Sala, J.M. Llovet, Gastroenterology **127**, S179 (2004). doi:10.1053/j.gastro.2004.09.032
5. K.T. Brown, J. Vasc. Interv. Radiol. **15**, 197 (2004). doi:10.1016/S1051-0443(07)60429-1

6. J.M. Llovet, M.I. Real, X. Montana, R. Planas, S. Coll, J. Aponte, C. Ayuso, M. Sala, J. Muchart, R. Sola, J. Rodes, J. Bruix, R. Vilana, L. Bianchi, J.R. Ayuso, T. Caralt, C. Bru, C. Acosta, M. Sole, Lancet **359**, 1734 (2002). doi:10.1016/S0140-6736(02)08649-X
7. T. Katsumori, K. Nakajima, T. Mihara, M. Tokuhiro, AJR. Am. J. Roentgenol. **178**, 135 (2002)
8. H. Nakamura, T. Tanaka, S. Hori, H. Yoshioka, C. Kuroda, J. Okamura, M. Sakurai, Radiology **147**, 401 (1983)
9. A. Laurent, R. Beaujeux, M. Wassef, D. Rufenacht, E. Boschetti, J.J. Merland, J.J. Am, Neuroradiology **17**, 533 (1996)
10. Y. Yokoi, S. Suzuki, T. Sakaguchi, T. Okumura, K. Kurachi, H. Konno, S. Nakamura, Radiat. Med. **20**, 45 (2002)
11. T. Katsumori, T. Kasahara, Cardiovasc. Intervent. Radiol. **29**, 1077 (2006). doi:10.1007/s00270-006-0059-y
12. K. Nilsson, F. Buzsaky, K. Mosbach, Bio/Technol. Nat. Biotechnol. **4**, 989 (1986). doi:10.1038/nbt1186-989
13. K. Ulubayram, E. Aksu, S.I. Gurhan, K. Serbetci, N. Hasirci, J. Biomater. Sci. Polym. Ed. **13**, 1203 (2002). doi:10.1163/156856202320892966
14. W.A. Bubnis, Anal. Biochem. **207**, 129 (1992). doi:10.1016/0003-2697(92)90513-7. C. M. 3rd. Ofner
15. E.M. Marks, D. Tourtellotte, A. Andux, Food Technol. **22**, 1433 (1968)
16. G.A. Digenis, T.B. Gold, V.P. Shah, J. Pharm. Sci. **83**, 915 (1994). doi:10.1002/jps.2600830702
17. C. Kuroda, M. Iwasaki, T. Tanaka, K. Tokunaga, S. Hori, H. Yoshioka, H. Nakamura, M. Sakurai, J. Okamura, Radiology **149**, 85 (1983)
18. T. Sonomura, Nippon Igaku Hoshasen Gakkai Zasshi **54**, 489 (1994)
19. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use, Q3C (R3): impurities: guideline for residual solvents (step 4 version, 2005) http://www.ich.org/LOB/media/MEDIA423.pdf. Accessed 11 Aug 2008
20. K. Ohta, Y. Matsuoka, Y. Ichikawa, K. Yamamoto, Oyo Yakuri **4**, 871 (1970)
21. G. Bryson, F. Bischoff, Prog. Exp. Tumor Res. **11**, 100 (1969)
22. K.A. Traul, A. Driedger, D.L. Ingle, D. Nakhasi, Food Chem. Toxicol. **38**, 79 (2000). doi:10.1016/S0278-6915(99)00106-4
23. S. Pestel, H.J. Martin, G.M. Maier, B. Guth, J. Pharmacol. Toxicol. Methods **54**, 200 (2006). doi:10.1016/j.vascn.2006.02.006
24. D.W. Cooper, Pharm. Eng. **16**, 1 (1996)
25. M.B. Gorbet, M.V. Sefton, Biomaterials **26**, 6811 (2005). doi:10.1016/j.biomaterials.2005.04.063