

Technical Note

Production and characterization of ECM powder: implications for tissue engineering applications

Thomas W. Gilbert^a, Donna Beer Stolz^b, Frank Biancaniello^c,
Abby Simmons-Byrd^d, Stephen F. Badylak^{a,*}

^a *Department of Surgery, McGowan Institute for Regenerative Medicine, University of Pittsburgh, 100 Technology Drive, Suite 200, Pittsburgh, PA 15219, USA*

^b *Center for Biological Imaging, University of Pittsburgh, Pittsburgh, PA 15213, USA*

^c *National Institute of Standards and Technology, Gaithersburg, MD, USA*

^d *ACell, Inc., West Lafayette, IN, USA*

Accepted 5 April 2004

Available online 24 June 2004

Abstract

Two methods to produce a particulate form of extracellular matrix (ECM) from porcine urinary bladder were investigated. One method to produce the powder involved snap freezing a lyophilized form of the material and then pulverizing it in a grinding mill. The second method was similar except that the ECM was saturated in a solution of NaCl prior to snap freezing to precipitate salt crystals within the matrix before grinding. Several methods were utilized to analyze the particle size distribution and ultrastructure including sonic sifting, laser diffraction, and scanning electron microscopy (SEM). The salt precipitation method yielded a more uniform distribution of particles with a smaller mean diameter (158 vs. 191 μm). SEM showed that the particles produced by grinding without salt precipitation were irregularly shaped, sheet-like particles. ECM particles produced by the salt precipitation method were round and porous in appearance with many particles in the range of 1 μm which tended to agglomerate with the larger particles and with each other. We conclude that the production of a comminuted form of ECM is possible and that the uniformity of particle size and shape are dependent upon the manufacturing methodology.

© 2004 Elsevier Ltd. All rights reserved.

Keywords: Extracellular matrix; Urinary bladder matrix; Powder; Particle size analysis; Scanning electron microscopy; Tissue engineering

1. Introduction

Naturally occurring extracellular matrix (ECM) has been shown to promote constructive remodelling as opposed to scar tissue formation in a variety of body systems, including but not limited to the lower urinary tract, esophagus, myocardium, arteries and veins, tendons and ligaments [1–8]. Most studies have used ECM that was not chemically crosslinked or otherwise altered by processing and that was isolated from porcine small intestinal submucosa or urinary bladder matrix (UBM). The single or multilayered sheet forms of these

materials have practical limitations with regard to their shape, three-dimensional form, and clinical utility. A particulate form of ECM is of interest for injection into tissues and for the development of three-dimensional scaffolds. The size and shape of the ECM particles may affect the host response to the particles in vivo. The size distribution and shape of the particles may differ depending upon the source and composition of the ECM and upon the method by which the powder is formed [9]. In the present study, UBM powder was formed by two methods: a first method by which the material was snap frozen in liquid nitrogen and then comminuted in a rotary knife mill, and a second method by which the material was saturated in a NaCl solution prior to snap freezing and grinding. The size of the particles was analyzed with the use of sonic sifting and a

*Corresponding author. Tel.: +1-412-235-5144; fax: +1-412-235-5110.

E-mail address: badylaks@upmc.edu (S.F. Badylak).

laser diffraction method. Scanning electron microscopy (SEM) was used to assess the ultrastructure of the particles.

2. Methods

The ECM was isolated from the porcine urinary bladder. The bladders were harvested immediately following euthanasia of market weight pigs (approximately 120 kg). The ECM isolation was performed by removing the tunica muscularis externa and tunica submucosa layers, leaving the basement membrane and tunica propria intact. The UBM was then washed in a 0.1% peracetic acid solution for 2 h with subsequent rinses in phosphate buffered saline and distilled water to disinfect the material and remove any cellular remnants.

Two methods were used to produce a particulate form of UBM. The first method involved lyophilizing the disinfected material and then chopping it into small sheets for immersion in liquid nitrogen. The snap frozen material was then reduced to small pieces with a Waring blender so that the particles were small enough to be placed in a rotary knife mill. A #60 screen was used to restrict the collected powder size to less than 250 μm .

The second method consisted of first soaking the disinfected material in a 30% (w/v) NaCl solution for 5 min. The material was then snap frozen in liquid nitrogen to precipitate salt crystals, and lyophilized to remove residual water. This material was then comminuted as described in the first method. By precipitating NaCl within the tissue, it was expected that the embedded salt crystals would cause the material to fracture into more uniformly sized particles. The particles were suspended in deionized water and centrifuged for 5 min at 1000 rpm three times to remove the NaCl. The suspension was snap frozen and lyophilized again. Finally, the powder was placed in a rotary knife mill to disaggregate the individual particles.

Sonic sifting and laser diffraction were used to analyze the particle size distribution that resulted from the two powdering methods. Sonic sifting involved separating the powder by size through a series of graduated screens stacked in a vertical configuration. The powder passed through the screens as a result of sonic pulses along the longitudinal axis of the stack and mechanical agitation in the plane of the screens. The screen sizes used were 212, 125, 90, 63, and 38 μm . The mass of the powder from each cut was weighed and the amount of material in each cut was represented as the percentage of the total mass. This data was converted to a percentage of the total volume based on an assumption that the density of the material was the same for the two production methods.

The laser diffraction method consisted of passing the dry particles through the path of a HeNe laser beam to

determine the powder size [10,11]. Each particle diffracted the light, and the diffraction angle was inversely proportional to the size of the particle. The diffraction pattern was detected using an array of detectors, and the particle size was calculated based on the angle and intensity observed.

The ultrastructure of the particles was analyzed using SEM. A thin layer of powder was mounted on adhesive metallic tape. The powder was sputter coated with 3.5 nm of gold. The samples were viewed at magnifications from $100\times$ to $500\times$.

3. Results

The results of particle size analysis using the sonic sifting technique on powder produced by the first method are presented in Fig. 1. The majority of the material was determined to be larger than 212 μm , with nearly 60% of the total volume not passing through the first screen. The fraction of particles with a size of less than 63 μm was estimated to be approximately 1% of the total volume. The mean particle size, based on an assumed maximum of 250 μm , was approximately 190.5 μm . However, it was noted that the particles tend to agglomerate on the screen despite the mechanical and sonic agitation. Therefore, the powder produced by this technique was also analyzed by laser diffraction. The average particle size as determined from the laser diffraction method was 158.0 μm . Based on laser diffraction, 18.7% of all particles were less than 63 μm , with 12.6% of particles less than 38 μm . While the results for the range of 63–125 μm were quite comparable between the two methods, the results for particles greater than 125 μm were quite different. Only 30% of the total volume of particles was found to be larger than 212 μm when measured by laser diffraction while nearly 60% were found to be larger than 212 μm when measured by sonic sifting. It was found that 90% of the particles were less than 336.7 μm (Fig. 2). This means

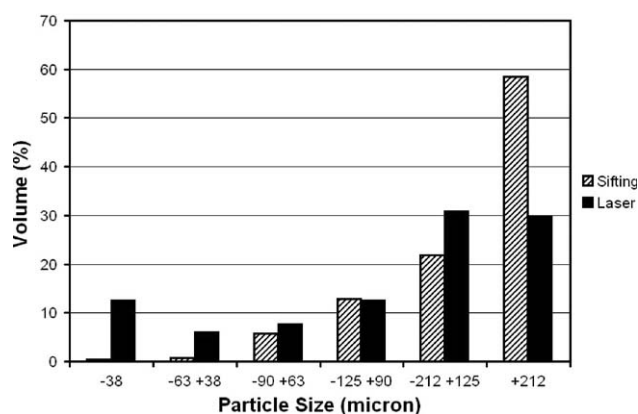


Fig. 1. Particle size distributions for UBM particles formed with a grinding method as analyzed by sonic sifting and laser diffraction.

that there were particles present with dimensions larger than the 250 μm mesh used to separate the particles during the grinding process.

The ultrastructure for particles produced by the first method was evaluated with SEM. The particles formed by the first method were irregularly shaped and could be defined generally as sheet-like or fiber-like (Fig. 3). Examination of the particles at higher magnification (500 $\times$) showed that two distinct surface ultrastructures were present (Fig. 4). One surface appeared to be quite smooth while the other appeared to be more fibrous.

Since sonic sifting did not give an accurate description of the particle size distribution, only laser diffraction was used to analyze the size of the UBM powder produced by the salt precipitation method. The mean

particle size was found to be smaller for powder produced by the salt precipitation method than the particle size produced by the first method (Fig. 2). Furthermore, the size of the particles was more uniform when produced by the salt precipitation method. The particles formed after salt precipitation showed a different morphology than those formed by the first method as shown with SEM. The larger particles in the distribution (150–200 μm) appeared to have porous surfaces and there was a large population of very fine

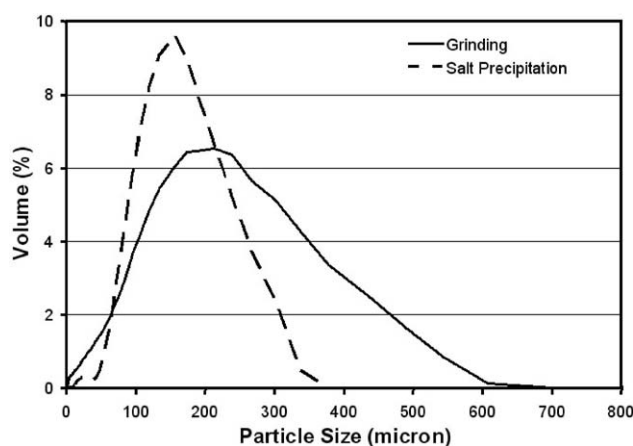


Fig. 2. Particle size distributions for UBM particles formed by the grinding and salt precipitation methods as analyzed by laser diffraction.

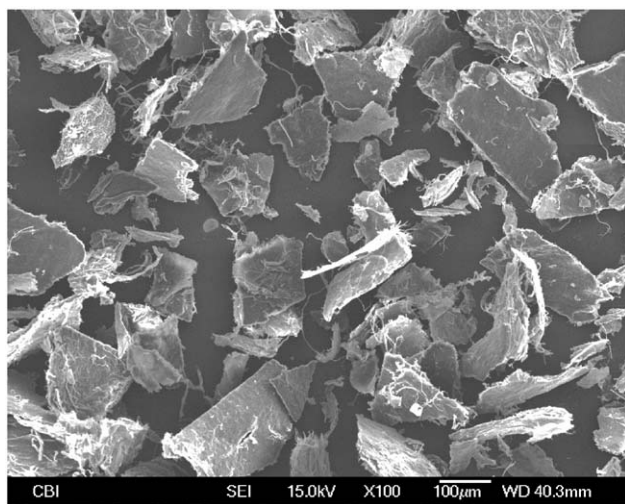


Fig. 3. Scanning electron micrograph of UBM particles produced by the first grinding method (100 $\times$). This material was sorted by sonic sifting through a 212 μm screen and collected on a 125 μm screen. Some of the particles are much smaller than the 125 μm cut-off, while others having dimensions that are much greater than 212 μm . This image also shows the irregular sheet-like shape of the particles.

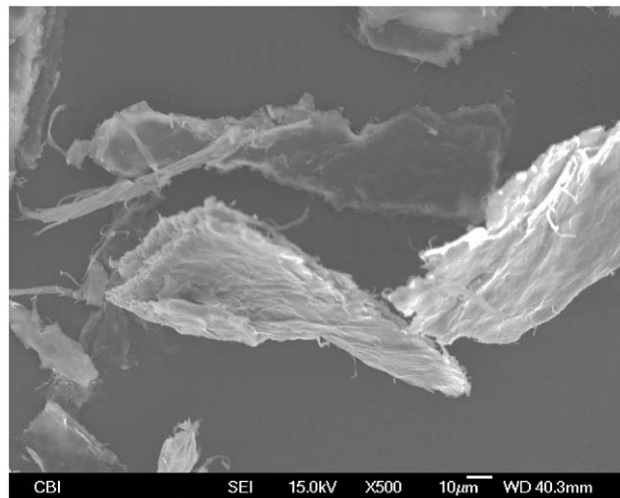


Fig. 4. Scanning electron micrograph of UBM particles produced by the first grinding method (500 $\times$). The two particles in the center of the image represent the two surfaces that were observed on all of the particles. It is thought that the smooth surface exhibited on the top particle is the basement membrane found on the sheet form of the material, while the textured, fibrous structure on the lower particle is representative of the tunica propria.

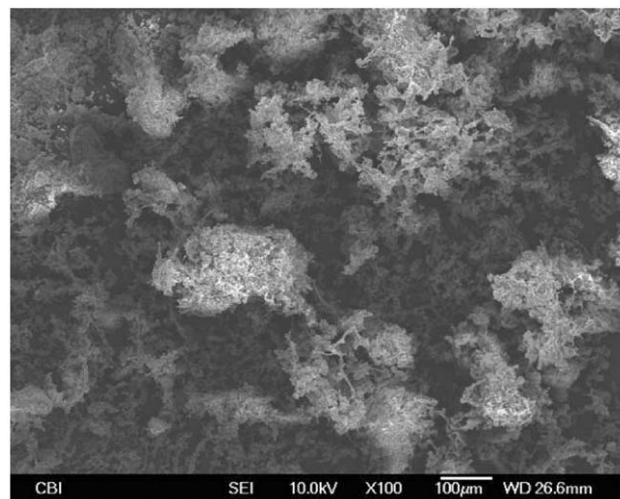


Fig. 5. Scanning electron micrograph of UBM particles produced by the salt precipitation method (100 $\times$). The population is characterized by larger particles which are rather porous in appearance and very small particles which tend to agglomerate with the larger particles and themselves.

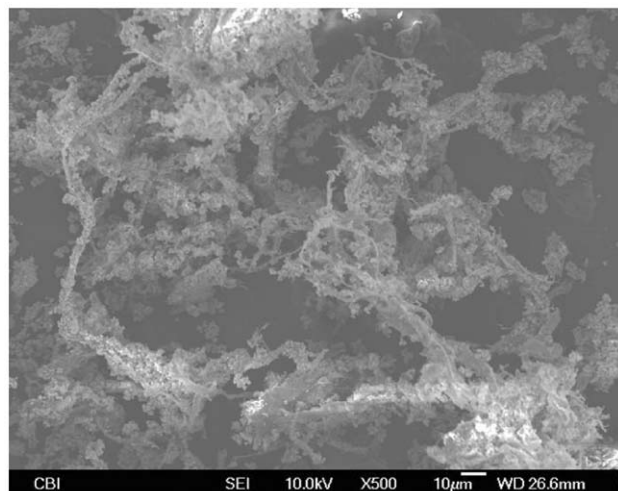


Fig. 6. Scanning electron micrograph of UBM particles produced by the salt precipitation method ($500\times$). Image presented small particles on the order of $1\mu\text{m}$ in diameter that are associated with collagen fibers.

particles ($\sim 1\mu\text{m}$) which tended to associate with the larger particles or agglomerate (Figs. 5 and 6).

4. Discussion

The current study investigated two techniques for producing UBM powder. Sonic sifting, laser diffraction, and SEM were found to be suitable to evaluate the particle size distribution and ultrastructure for the material in this study [12]. Sonic sifting did not give a representative distribution of the particle size due to agglomeration of the particles. Furthermore, due to the irregular shape of the particles, the result only reflected the smallest dimension of the particle in many cases. This observation was confirmed by SEM. Laser diffraction was more sensitive for detecting smaller particles and was better able to establish an upper bound for the particle size distribution compared to sonic sifting. Comparing the two powdering techniques, the salt precipitation method produced smaller particles that were more uniformly distributed than those obtained by the first method.

The irregular shape and size of the particles is likely due to the fibrous nature of this largely collagenous material. The grinding process is more likely to shear the material between adjacent collagen fibers than it is to actually cut a fiber along its length. Individual fibers are visible both alone and associated with other particles (Fig. 3).

SEM also showed differences in the powder surface when the two methods of comminution are compared. The different surface appearances observed on particles produced by the first method are consistent with the

sidedness of the original sheet form of the material with the basement membrane (smooth) on one side and the tunica propria (fibrous) on the other. The basement membrane may contribute to the formation of the sheet-like particles, with the dense matrix holding the fibrous tunica propria together. The particles produced by the salt precipitation method appeared to be agglomerations of smaller particles, which may have limited the ability of the laser diffraction to accurately measure the particle size. The larger particles produced by salt precipitation were more porous and were often associated with or attached to very small particles (Fig. 5). The difference in size and morphology could have an effect on how cells interact with the matrix.

Naturally occurring ECM has been shown to support the constructive remodelling of damaged or missing tissues in adult mammals. More specifically, scar tissue tends to be minimal while site specific tissue remodelling occurs [2–4, 6, 8, 13, 14]. The constructive remodelling process is due, in part, to bioactive peptides released during degradation that attract circulating bone marrow derived progenitor cells [13], and to the presence of active growth factors resident in the matrix such as VEGF, bFGF, and TGF- β [15,16]. Therefore, the means to produce ECM powder and expand the potential clinical applications of this material may be important for the field of tissue engineering. The concept of using ECM powder with bioinductive properties has been utilized extensively for the delivery of demineralized bone matrix in periodontal and orthopaedic applications [17–19]. A powdered form of ECM provides a great deal more flexibility in terms of delivery of the material to target sites. For example, an ECM powder could be administered locally via a syringe and needle, whereas this would not be possible with sheet forms of the material. For an injectable form of ECM powder to be a reality, a uniformly small particle size will be needed so the particles do not obstruct the syringe needle lumen. The powder described in the current study has been successfully injected through a 22 gauge needle ($410\mu\text{m}$ inner diameter), which is a typical size used for intramuscular injection. However, higher gauge needles pose more of a challenge since a 25 gauge needle only has an inner diameter of $250\mu\text{m}$. In addition to facilitating delivery of the ECM in a clinical, minimally invasive procedure, a powdered form of the ECM also makes possible the development of three-dimensional scaffolds for the reconstruction of more complex tissue structures. Additional study is needed to investigate the mammalian tissue response to a particulate form of ECM and to examine the effect of particle size upon such a device [9].

The present study shows that the particle size distribution and morphology is highly dependent on the method of production. ECM particles produced solely by grinding were irregularly shaped, sheet-like,

and retained the ultrastructure observed on the sheet form of the material. With the addition of salt precipitation, the particles were smaller and more uniform in size and appeared to be more porous. Additional work is needed to investigate the response of mammalian tissues to ECM powder, as well as to further refine the production techniques for clinical use.

Acknowledgements

We would like to acknowledge funding from the NIH (EB000261) and the original work of Dr. Tim McPherson.

References

- [1] Badylak SF, Lantz GC, Coffey A, Geddes LA. Small intestinal submucosa as a large diameter vascular graft in the dog. *J Surg Res* 1989;47(1):74–80.
- [2] Badylak SF, Tullius R, Kokini K, Shelbourne KD, Klotwyk T, Voytik SL, et al. The use of xenogeneic small intestinal submucosa as a biomaterial for Achilles tendon repair in a dog model. *J Biomed Mater Res* 1995;29(8):977–85.
- [3] Badylak S, Meurling S, Chen M, Spievack A, Simmons-Byrd A. Resorbable bioscaffold for esophageal repair in a dog model. *J Pediatr Surg* 2000;35(7):1097–103.
- [4] Badylak S, Obermiller J, Geddes L, Matheny R. Extracellular matrix for myocardial repair. *Heart Surg Forum* 2003;6(2):E20–6.
- [5] Vaught JD, Kropp BP, Sawyer BD, Rippey MK, Badylak SF, Shannon HE, et al. Detrusor regeneration in the rat using porcine small intestinal submucosal grafts: functional innervation and receptor expression. *J Urol* 1996;155(1):374–8.
- [6] Kropp BP, Rippey MK, Badylak SF, Adams MC, Keating MA, Rink RC, et al. Regenerative urinary bladder augmentation using small intestinal submucosa: urodynamic and histopathologic assessment in long-term canine bladder augmentations. *J Urol* 1996;155(6):2098–104.
- [7] Kropp BP, Eppley BL, Prevel CD, Rippey MK, Harruff RC, Badylak SF, et al. Experimental assessment of small intestinal submucosa as a bladder wall substitute. *Urology* 1995;46(3):396–400.
- [8] DeJardin LM, Arnoczky SP, Clarke RB. Use of small intestinal submucosal implants for regeneration of large fascial defects: an experimental study in dogs. *J Biomed Mater Res* 1999;46(2):203–11.
- [9] Sampath TK, Reddi AH. Importance of geometry of the extracellular matrix in endochondral bone differentiation. *J Cell Biol* 1984;98(6):2192–7.
- [10] Barber D, Keuter J, Kravig K. A logical stepwise approach to laser diffraction particle size distribution analysis methods development and validation. *Pharm Dev Technol* 1998;3(2):153–61.
- [11] Kleinebudde P, Jumaa M, El Saleh F. Influence of the degree of polymerization on the behavior of cellulose during homogenization and extrusion/spheronization. *AAPS PharmSci* 2000;2(3):E21.
- [12] Barth H. Modern methods of particle size analysis. New York: Wiley; 1985.
- [13] Badylak SF, Park K, Peppas N, McCabe G, Yoder M. Marrow-derived cells populate scaffolds composed of xenogeneic extracellular matrix. *Exp Hematol* 2001;29(11):1310–8.
- [14] Huber JE, Spievack A, Simmons-Byrd A, Ringel RL, Badylak S. Extracellular matrix as a scaffold for laryngeal reconstruction. *Ann Otol Rhinol Laryngol* 2003;112(5):428–33.
- [15] Hodde JP, Record RD, Liang HA, Badylak SF. Vascular endothelial growth factor in porcine-derived extracellular matrix. *Endothelium* 2001;8(1):11–24.
- [16] Voytik-Harbin SL, Brightman AO, Kraine MR, Waisner B, Badylak SF. Identification of extractable growth factors from small intestinal submucosa. *J Cell Biochem* 1997;67(4):478–91.
- [17] Mizuno S, Glowacki J. Three-dimensional composite of demineralized bone powder and collagen for in vitro analysis of chondroinduction of human dermal fibroblasts. *Biomaterials* 1996;17(18):1819–25.
- [18] Maddox E, Zhan M, Mundy GR, Drohan WN, Burgess WH. Optimizing human demineralized bone matrix for clinical application. *Tissue Eng* 2000;6(4):441–8.
- [19] Tsai CH, Chou MY, Jonas M, Tien YT, Chi EY. A composite graft material containing bone particles and collagen in osteoinduction in mouse. *J Biomed Mater Res* 2002;63(1):65–70.