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Short communication

Floating porous alumina from protein foaming-consolidation technique for cell culture application

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Abstract

Floating porous ceramics have been successfully fabricated using protein foaming-consolidation method. Alumina powder and yolk as the pore creator were mixed while stirring and the resulting slips were poured into cylindrical shaped molds followed by drying for foaming-consolidation process. The dried green bodies were then burned at 600 °C for 1 h to remove the pore creating agent and finally sintered at 1550 °C for 2 h. The sintered alumina ceramics with shrinkage in the range of 29–40 vol.% and pore sizes of 250–500 μm were obtained. The compressive strength of sample was 1.1 at 70.6% porosity and it increased to 1.7 MPa at 52.8% porosity. The density varied in the range of 0.8–1.1 g/cm^3 depending on the composition of the slurry. Preliminary cytotoxicity test of the samples conducted using DF-1 cell culture showed promising results.

Keywords: Floating; Porous ceramic; Protein foaming-consolidation; Cell culture

1. Introduction

Cell culture is one method used for producing many biological materials such as vaccines, enzymes, hormones, antibodies and nucleic acids [1]. Microcarrier culture technique is one of the methods developed for cell cultivation. Owing to high surface, microcarrier culture offers a practical high yield culture of anchorage-dependent cells. In microcarrier culture cells grow as monolayer on the surface of small spheres or as multilayers in the pores of macroporous structures that are usually suspended in culture medium by gentle stirring [1,2]. Several factors are crucial for the successful application of microcarriers in tissue engineering. The attachment of cells to the microcarrier surface depends on the chemical composition, surface topography, degree of porosity and charge density. In addition, the number of cells attachable on the surface depends on the carrier diameter (typically in the range of 100–400 μm). The size distribution should be as narrow as possible to provide a homogeneous culture, and the specific density of the microcarrier beads should be slightly higher than that of the culture medium (typically between 1.02 and 1.10 g/cm^3) to allow floatability in suspension while being agitated [2].

The performance of microcarrier will be depending on many parameters including chemical, physical and geometrical properties of the carrier. A wide variety of materials such as gelatine, silica, collagen and glass have been utilized to produce microcarriers [3]. However, cell growth on these microcarriers in a stirred bioreactor is low due to high microcarrier density, possibly leading to nonuniform cell spreading. Some requirements of microcarrier are needed to optimize cell production, *i.e.* floatable on culture medium, biocompatible and high resistance on temperature, pH and mechanical vibration. Microcarriers with high thermal resistance are needed to avoid its body damage when autoclaved at high temperature (normally up to *ca.* 120 °C). Porous ceramics are predicted to meet the special necessities of a microcarrier technique such as good mechanical, chemical and thermal resistances [1]. There are a large number of methods to produce porous ceramics as discussed in the literature [1]. Some prominent methods include freeze casting [4], electrophoretic deposition [5] and gel casting [6,7] that use a variety of pore-forming agents like glycerol [4], agarose [6] and camphene [8].

Recently, we have developed protein foaming-consolidation method using egg yolk as the pore creating agent to produce

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porous ceramics [9]. In this report, floating porous alumina was prepared through this technique. The effect of slurry composition on the physical properties of the obtained porous ceramic bodies was investigated.

2. Experimental

Alumina powder (Sigma Aldrich, USA) and yolk (freshly isolated from chicken egg) were mixed in a beaker glass with alumina-to-yolk mass ratios of 0.65 (slurry A), 0.75 (slurry B) and 1.00 (slurry C). Darvan 821 A (R.T. Vanderbilt, USA) with concentration of 0.01 wt.% was added into the slurry C only. The slurries were magnetically stirred with 150 rpm rate for 3 h at room temperature. The slurries were cast into cylindrical open stainless steel molds and heated in an air oven (Memmert, 100-800 model) at 180 °C for 1 h. Castor oil was used as lubricant for easy demolding. The dried samples were heated in a furnace (Protherm, PLF 160/5 model) at 10 °C/min rate up to 600 °C for removal of the yolk and then at 2 °C/min rate up to 1550 °C.

Foaming capacity of slurry was evaluated by measuring the change in volume of slurry during drying process. The foaming capacity is calculated in terms of the volume ratio of foamed slurry to the original one. The rheological property of the slurries was measured in a ThermoHaake VT 550 rheometer with a measuring system of concentric cylinders sensor cone of SV-DIN type. The density and total porosity of sintered specimens were obtained using the Archimedes method with a theoretical density of 3.98 g/cm^3 for alumina. The micro and macrostructure observation of specimens were carried out on a JEOL 6700 F FESEM. CT-scan (Xradia, μXCT model) was used to observe 3D internal structures of porous alumina.

The *in vitro* cytotoxicity was investigated using DF-1 cells culture. DF-1 cells obtained from Cell and Tissue Engineering Laboratory of IIUM were grown in 15 mL fresh Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). This test was carried out in 75 cm^3 culture T-flaks at 37 °C and 5% CO_2. After confluence, the cells were detached with 0.05% trypsin. Subsequently, DF-1 cells were seeded on the alumina porous body (2 mm × 1 mm × 1 mm dimension) and media in 96-well plate. The morphology and spreading of cells on the specimen were assessed by SEM (JEOL, 5600 model) measurement after 5 days incubation. The specimens were fixed with 4% glutaraldehyde for 30 min and dehydrated in solutions containing ethanol of various concentrations (10%, 30%, 50%, 70%, 90%, and 100%) before 37 °C drying in air over night.

3. Results and discussion

Analyses of slurries viscosity were carried out within 3 h after mixing at ambient temperature and measured at shear rates ranging from 10 to 500 s^{-1} as shown in Fig. 1. The slurries with alumina-to-yolk mass ratios of 0.65 (slurry A) and 0.75 (slurry B) show pseudoplastic flow behavior, whereas the slurry C shows Newtonian fluid. At low shear rate (10 s^{-1}), the slurries

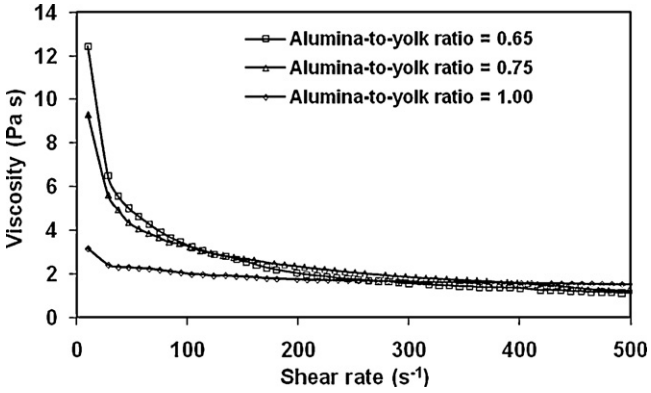

Fig. 1. Rheology of slurries with different alumina-to-yolk mass ratio.

A and B show large difference in viscosity (9.3–12.4 Pa s), but the viscosity values are very close as shear rate is high. The average viscosity value of the slurries at high shear rate (500 s^{-1}) is 1.2 Pa s indicating that the slurries are pourable under shear. The addition of dispersant (darvan 821 A) with concentration of 0.01 wt.% into the slurry C shifted the rheological properties from pseudoplastic behavior to Newtonian fluid.

To describe the rheological behavior of slurries, the measured data of viscosity was fitted with a power law model, $\eta = k\gamma^{n-1}$, where η is the viscosity of the slurry, γ is the applied shear rate, k and n are the consistency factor and non-Newtonian index, respectively [6]. The calculated n index for slurries A, B, and C are 0.32, 0.41 and 0.81, respectively. Increasing n values from 0.32 to 0.81 indicated that the rheological behavior transformed from shear thinning flow to Newtonian fluid. On the other hand, the calculated k parameter significantly decreased from 74.2 to 3.9 when alumina-to-yolk ratio was increased from 0.64 to 1.00. Although slurry C contains high solid content, its viscosity is the lowest showing a Newtonian fluid behavior even after dispersant addition as low as 0.01 wt.%.

Fig. 2 presents the effect of slurry composition (slurries A, B and C) on the foaming capacity after 1 h drying time. The foaming capacity decreased with the increasing alumina-to-yolk mass ratio in initial slurry. The foaming capacity of slurry A (0.65 alumina-to-yolk mass ratio) and B (0.75 alumina-to-yolk mass ratio) are 2.2 and 1.9, v/v, respectively. The foaming

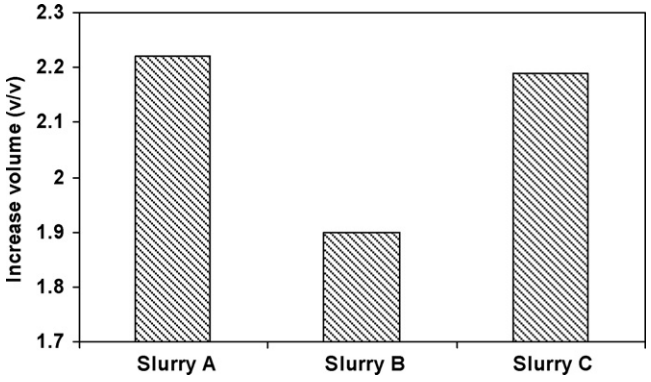

Fig. 2. The foaming capacity of slurries A, B and C after dried for 1 h.

Table 1
Characteristics of sintered alumina samples.

Slurry	Alumina-to-yolk mass ratio	Shrinkage (vol.%)	Density (g/cm^3)	Porosity (%)	Strength (MPa)
A	0.65	39.9	0.87	70.6	1.1
B	0.75	36.4	1.11	52.8	1.7
C[a]	1.00	31.5	0.85	78.5	1.4

[a] The slurry contains dispersant "Darvan 821 A" of 0.01 wt.%.

agent of the slurry is protein in the egg yolk. The physical and chemical properties of protein would alter (called denaturation) when the slurry is dried at 180 °C. The protein foams due to their amphiphilic characters [10]; the increasing yolk content increased the foaming ability of slurry.

In general, viscosity is known to affect both the stability of the foam and its foaming ability [11]. The foaming capacity of slurry C (1.00 alumina-to-yolk ratio) is higher (2.19, v/v) than slurry B (0.75, w/w, alumina-to-yolk ratio) indicating that although slurry C containing lowest yolk, but addition of dispersant has improved its foaming ability. It could be deduced that dispersant molecules decreased the viscosity of slurry (see Fig. 1) and accelerated transfer proteins from interior of the slurry toward the newly created surface, thus reducing the surface tensions and increasing the foaming capacity.

The sintering shrinkage of the samples of 31.5–39.9 vol.% was observed and it increases as the portion of yolk in the green body is high. Porosity of the samples prepared from slurry composition from A to C is in the range of 52.8–78.5%, and increases as the amount of pore former increases. The sintering shrinkage and porosity value of each sample is summarized in Table 1. The table also presents compressive strength of each porous alumina. The compressive strength of porous alumina samples (without dispersant addition) decreases from 1.7 (sample B) to 1.1 MPa (sample A) when porosity increases from 52.8 to 70.6%.

The porous alumina bodies show less cracks during burn out of pore former and sintering. The density of obtained porous alumina was in the range of 0.85–1.11 g/cm^3 and they show floatability on water as shown in Fig. 3a. Three dimensional (3D) image of sample A measured using micro-CT scan is presented in Fig. 3b. The pore interconnectivity and struts like structure of bone can easily be seen in the figure. In bone implant application, interconnected pores permit tissue and bone ingrowth, preventing loosening and at the same time retaining dynamic strength of implants [12].

FESEM observation on sample A shows that the pore size is in the range 250–500 μm (Fig. 4a). Dimension and morphology of pores are crucial factor for an excellent osteointegration. Reportedly, pore size should be 200–500 μm for colonization of osteoblast in the pores, fibrovascular ingrowth and finally the deposition of the new bone [1].

As the alumina content decreased more pores are found, thus showing poorer densification of particles (Fig. 4b). Low alumina content likely resulted in low viscosity, which corresponded to high foaming capacity and low density. Conversely, when alumina loading increased (slurry C) the walls of porous body became denser as shown in Fig. 4c. Moreover, the grain size of porous alumina walls shows irregularity and in some parts small particles adsorb on the large grains. There is more bonding area among grains which may probably become the origin of higher strength of the porous ceramics.

To confirm that fabricated porous alumina is non-toxic even after sintering step, cytotoxicity study was conducted in tissue culture. Culture media changed from purple to yellow color after 5 days incubation time, indicating excellent cells growth. Alteration of media color is caused by actively growing cells which have produced lactic acid that was released into the media. Fig. 5 shows the SEM micrograph of the alumina sample that was seeded with DF-1 cells. It can be observed that the cells attach and spread over the alumina surface (Fig. 5a). The cells

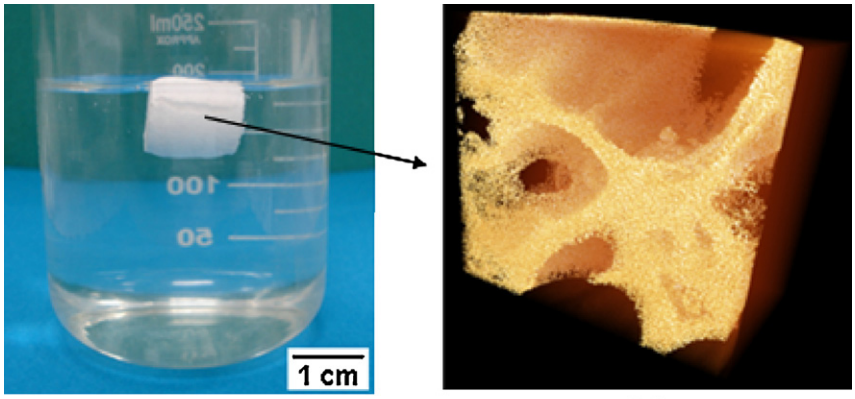

Fig. 3. (a) A porous alumina floats on water and (b) CT-scan image of the body.

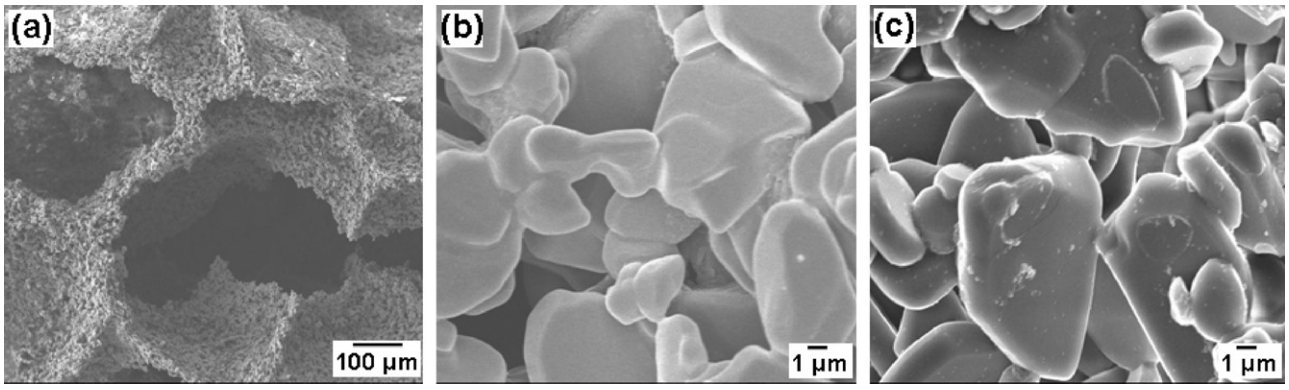

Fig. 4. Macro- and microstructure of porous sample 0.65 alumina-to-yolk mass ratio (a, b) and microstructure of porous sample 1.00 alumina-to-yolk mass ratio (c).

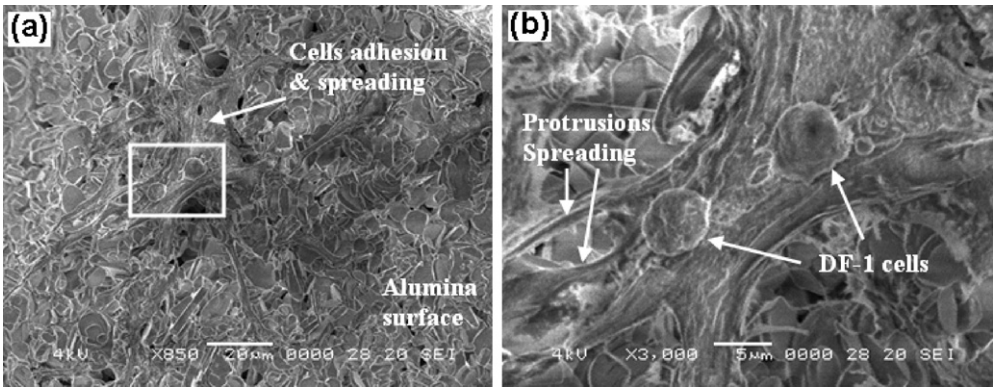

Fig. 5. SEM images of the DF-1 cells on the porous alumina: (a) top surface and (b) enlarged area of (a).

exhibit a dense layer (Fig. 5b) due to highly active proliferation, a characteristic supported by the rounded shape of the DF-1 cells. This morphology is typical for cells that grow in monolayer, energetically favorable to the cell division process. During the cell growth phase, they show an accelerated metabolism, because they are susceptible to pH, temperature, PO_2 and pCO_2 variations [13].

Porous alumina ceramics produced by the current method are floatable on water or any common culture media as its density in the range of 0.85–1.11 g/cm^3. It also showed high compressive strength and non-toxicity. Therefore, the porous alumina can be a potential candidate for floating microcarrier application, especially in a bioreactor cell culture. The main advantage of the porous ceramic microcarrier is cells of 20–30 μm sizes can be entrapped in pores with average diameter of 250–500 μm and grow without damage by fluid turbulence and vibration. With enlarged pore channels that connect pores, cells can easily permeate into it and thus 3D cell cultivation is possible.

4. Conclusions

A protein foaming-consolidation technique has been utilized to manufacture floating porous alumina ceramics using egg yolk as the pore creating agent. The physical properties of samples can be controlled by varying slurry compositions. Addition of yolk in slurry decreased the viscosity and increased the foaming capacity. The density of obtained porous alumina was in the range of 0.8–1.1 g/cm^3 with the compressive strength of 1.1–1.7 MPa. The sintered porous bodies have open, interconnected porous structure with pore size of 250–500 μm. When the alumina-to-yolk increased from 0.65 to 0.75, the porous bodies shrunk causing a porosity decrease, which in turn led to improved compressive strength. From the preliminary *in vitro* test, it was found that the porous materials are non-toxic and provide favorable sites for attachment and growth of DF-1 cells.

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