Surfactant-Free Poly(lactide-co-glycolide) Honeycomb Films for Tissue Engineering: Relating Solvent, Monomer Ratio and Humidity to Scaffold Structure

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ABSTRACT

Here we introduce a one-step surfactant-free water droplet templating as a fabrication method for a poly(lacitide-co-glycolide) (PLGA) film that can be used as a model to investigate the relationship between solvent, monomer ratio, polymer concentration and humidity on its structure; the resulting structure is a honeycomb structured film. Formation of the honeycomb structure was highly sensitive to solvent, monomer ratio, polymer concentration and humidity. The results show surfactant-free water droplet templating allows investigation of fabrication parameters, and that PLGA monomer ratio selection is important for scaffold structure but not for MG63 cell attachment and proliferation.

Keywords: PLGA, scaffold, tissue engineering, water droplet templating

Introduction

Poly(α-hydroxy acids) such as polylactide (PLA), polyglycolide (PGA) and their copolymer poly(lactide-co-glycolide) (PLGA) are some of the most widely used synthetic polymeric materials for scaffold fabrication (Rose and Oreffo 2002). A particularly versatile yet simple and cheap scaffold fabrication technique, using mild conditions, is phase inversion membrane casting. It has been shown that membrane morphology is dependent on many physical and chemical factors, including monomer ratio, molecular weight and polydispersity index (Ellis and Chaudhuri 2010), as well as the properties of the polymer solution and the nonsolvent (Ellis and Chaudhuri 2007). However, further understanding of the process parameters is necessary to accurately determine the appropriate casing conditions. Another example of phase inversion is the water droplet method, using condensed water droplets as template to microporous thin films with highly ordered honeycomb-shaped pores. Francois and co-workers reported that star-like polystyrene and polystyrene-polyparaphenylene in carbon disulphide can be fabricated into honeycomb structured thin films with highly ordered pores by evaporation of the polymer solutions under a flow of moist gas (Widawski *et al.* 1994). The proposed mechanisms have been previously described (Stenzel et al. 2006; Bunz 2006) and involves solvent evaporation, water droplet condensation on the polymer solution surface, formation of close-packed arrays of water droplets, and polymer precipitation at the solvent-water droplet interface. Similar honeycomb structured PLA and its copolymers have also been reported (Fukuhira *et al.* 2006; Zhao *et al.* 2006) but inclusion of a surfactant was considered as an essential additive to stabilize the water droplets and to act as a template for honeycomb formation. However, the toxicity and metabolic pathway of the surfactants are still poorly understood and as such their presence is undesirable.

This study demonstrates that honeycomb structured films can be fabricated from PLGA copolymers without the need for surfactants. Additionally the system is highly sensitive to conditions so it can be used as a tool to investigate the relationship between process parameters. The influence of solvent, relative humidity and concentration on pore formation were investigated. Osteoblast-like MG63 cells were cultured on the PLGA thin films to evaluate their suitability as scaffolds.

Materials and Methods

Materials

PLGA with nLA:nGA=85:15 and 75:25 (Lakeshore Biomaterials Inc.) and nLA:nGA=50:50 (Aldrich) were all GMP grade. Chloroform ($CHCl_3$, HPLC grade), tetrahydrofuran (THF, GPC grade) and ethyl acetate (HPLC grade) were all purchased from Fisher scientific and used as received without further purification unless otherwise stated. Cell culture reagents such as Dulbecco's Modified Eagles Medium (DMEM), heat inactivated foetal calf serum (FCS), sodium pyruvate, antibiotic-antimycotic and non-essential amino acid (NEAA), phosphate buffered saline (PBS) were purchased from Sigma-Aldrich.

Measurement of polymer molecular weight

The average molecular weight of polymers were analysed by Gel Permeation Chromatography (GPC). The analyses were performed on a Polymer Laboratories PL-GPC50 integrated system using a PLgel 5 μm MIXED-D 300×7.5 mm column at 35 °C. THF was used as the mobile phase at a flow rate of 1.0 ml/min. The measured molecular weight for the polymers were obtained: PLGA50:50 ($\overline{Mw}$ =53,000 g·mol^{-1} $\overline{Mn}$ =34,000 g·mol^{-1}), PLGA75:25 ($\overline{Mw}$ =290,000 g·mol^{-1} $\overline{Mn}$=190,000 g·mol^{-1}) and PLGA85:15 ($\overline{Mw}$=120,000 g·mol^{-1} $\overline{Mn}$=57,000 g·mol^{-1})

Film preparation and characterization

The films were prepared in a closed box with defined temperature and humidity. The relative humidity was controlled by adjusting the air flow rate through a two-necked flask containing distilled water. The polymer solution (200 μl) was cast onto 13 mm diameter glass cover slips under humid conditions. The morphology of thin films was characterized by scanning electron microscopy (JOEL JSM 6480LV SEM) with 10-15 kV accelerating voltage and pore size was analysed using the ImageJ software package (Wei *et al.* 2007)

Cell culture on PLGA films

MG63 Osteosarcorma cell line (European Collection of Cell Culture, ECACC) were used to assess cell viability on the honeycomb structured films. Silicon rings were placed on the top of the PLGA films in order to prevent detachment of the thin films from cover slips. The thin films were sterilised by submerging in antibiotic-antimycotic solution (1% in PBS) over night and washed twice with excess PBS prior to cell culture. The cells were seeded at a concentration of 40,000/ml onto the thin PLGA films which were placed in tissue culture treated 24-well plates (Nuclon^{TM} Δ Surface) and maintained in a humidified environment at 37°C and 5% CO_2. Cells were maintained in culture medium which contained DMEM, supplemented with 10% (v/v) FCS, 1 mM sodium pyruvate 1% (v/v) antibiotic-antimycotic and 1% (v/v) NEAA. The MG63 cells seeded on the tissue culture treated polystyrene (TCPS, Nuclon^{TM} Δ Surface) without PLGA films were used as positive control.

Cell viability and morphology

Cell viability on the PLGA films and TCPS were determined by using a Sigma TOX-2 XTT assay kit according to the manufacturer's instructions. The optical density of the resulting dye was measured with 490nm test wavelength and a 630 nm reference wavelength using a plate reader (Synergy^{TM} HT, BioTek). The results were shown as a percentage of the control. The data were expressed as mean values ± standard deviation (s.d.). Statistical comparisons were carried out using two-way analysis of variance (two-way ANOVA) with Bonferroni post tests. Results were considered statistically significant at $p<0.05$. All the analyses were performed with GraphPad Prism 5.0 software. Cell morphology was examined at day 3 by scanning electron microscopy (JOEL JSM 6480LV SEM).

Results and Discussion

The properties of the solvent determines honeycomb formation

Solvent properties, such as water miscibility, evaporation rate, and density strongly affect honeycomb film formation (Stenzel *et al.* 2006). For instance, Peng and co-workers reported that

honeycomb structured non-end-group functionalized polystyrene (PS) can be fabricated using $CHCl_3$ as the solvent but not from carbon disulphide (CS_2) and THF due to the impact of evaporation rate on pattern formation. In this study, water miscible solvent THF and the water immiscible solvents, $CHCl_3$ and ethyl acetate, with different volatility were selected to investigate the influence of solvents on the formation of honeycomb PLGA films *(*Peng *et al. 2004)*. Figure 1 shows SEM images of films prepared from PLGA50:50, PLGA75:25 and PLGA85:15 copolymers using the water droplet templating technique. Honeycomb structured PLGA50:50 and PLGA75:25 films were generated from $CHCl_3$, and the average pore diameter was recorded as 4.2 ± 0.8 μm and 4.3 ± 0.7 μm respectively (Figure 1a & d). However, the films prepared from PLGA85:15/$CHCl_3$ system (Figure 1g) did not show regular patterns on the surface; it exhibited various pore sizes ranging from ~ 4.5 to 50.5 μm in diameter. These observations were consistent with increasing glycolic content leading to an increase in polymer hydrophilicity, which has previously been reported to favour the formation of honeycomb films of PLGA from $CHCl_3$ (Zhao *et al.* 2003).

On the other hand, no honeycomb films were formed with ethyl acetate, regardless of the LA:GA ratio (Figure 1b, e & h). It was thought the volatilization rate of ethyl acetate played important roles in the process of fabrication. The vapour pressure for ethyl acetate and $CHCl_3$ at 20ºC is 10 kPa and 21.28 kPa, respectively (Lide 2005; Peng et al. 2004). Lower vapour pressure usually leads to a slower evaporation (i.e. ethyl acetate evaporates slower than $CHCl_3$ at the same conditions). During the ethyl acetate evaporation process, water droplets condensed onto the polymer-air interface due to cooling effect of the evaporation. However, the convention force caused by temperature gradient due to slow evaporation of ethyl acetate may not strong enough to suppress coalescence of small water droplets into large droplets before the solvent evaporated completely. Therefore PLGA/ethyl acetate system is unable to stabilize water droplets, which in turn leads to random distribution of the pore sizes.

Usually it is considered that water miscible solvents (e.g. THF) are not beneficial to stabilize water droplets due to its miscibility with water (Zhao *et al.* 2006). However, Park and co-workers (Park and Kim 2004) showed that cellulose acetate butyrate (CAB) films with hexagonal packed pores

can be prepared from THF. The authors suggested that the hydrophilicity of the CAB chains were crucial in stabilization of water droplets. In this study, all the PLGA copolymers were not able to generate honeycomb structures from THF with 1 g/l concentration (Figure 1c, f & i). The pore size of the resulting films varied from ~2.5 to 20.5 μm. On the other hand, Zhao et. al. (Zhao *et al.* 2003) successfully prepared honeycomb structured poly(L-lactide) films from THF, at 50 g/l polymer concentration, which indicates that the concentration of the polymer solution has a dramatic influence on honeycomb pattern formation.

Optimization of honeycomb patterns by varying polymer concentration

The effect of polymer concentration on the honeycomb pattern formation was investigated by varying the concentration of PLGA in THF and $CHCl_3$ at 90 % humidity. A clear trend for PLGA85:15 on honeycomb film formation was seen (Figure 2k-o) at low concentration (1 g/l), the film exhibited a broad pore size distribution (14.5 ± 8.0 μm). It started to form a honeycomb pattern but still with a number of non-circular pores when the concentration increased to 10 g/l. The honeycomb structures were optimised at concentration of 20 and 25 g/l and resulted in honeycombs with average pore diameter 4.2 ± 0.3 and 3.7 ± 0.2 μm. With a further increase of concentration (30 g/l), the pores began to distort again. This dramatic change of the morphologies could be attributed to the change of viscosity and surface tension in the solution. The viscosity of the PLGA85:15 solution was too low to prevent water droplets coalescing at 1 g/l. The combination of weakened thermocapillary convection,f greater surface tension and viscous polymer solution at higher concentration, which facilitated the stabilization of water droplets (Karikari *et al.* 2006). However, the water droplets were not able to penetrate into the polymer interfacial layer if the concentration was too high. Hence, it led to only a few pores or no pore formation on the film. Similar trends, but lower concentration range, were observed in PLGA50:50 and PLGA75:25/$CHCl_3$ system as shown in Figure 2(a-e) and Figure 2(f-j). For PLGA50:50 and PLGA75:25 the optimised honeycomb patterns were obtained at concentrations of 1 g/l and 3 g/l. The average pore diameter decreased with increasing polymer concentration. The thickness of the walls between pores increased with increasing polymer concentration. At 10 g/l, there were no

pores on the PLGA50:50 and only a few distorted pores on the PLGA75:25. It showed that polymer with too high concentration prevented the water droplets to penetrate into the solution surface.

Pore size is affected by the humidity

To investigate the influence of humidity on the pore size of honeycomb films, PLGA50:50 /$CHCl_3$ (1 g/l), PLGA75:25/$CHCl_3$ (1 g/l) and PLGA85:15/THF (20 g/l) solutions were applied to prepare the films. The humidity was varied from 50% to 90%. It can be seen from Figure 3 that pore size was closely related to the humidity. The regularity of the pattern formation improved as humidity increased from 50% to 90%. At 90% humidity, the pattern formation was more well-controlled compared to the ones fabricated at lower humidity. However, there was no pore formation on all the PLGA films when the humidity was 40%. Park and co-workers were also observed that there was no pore formation at 40% humidity when evaporated 0.05 g/ml CAB from THF (Park and Kim 2004). At low humidity condition, water droplets did not condense on the polymer solution. This provided evidence that moisture content was critical in the water droplets templating method.

Honeycomb films supported more MG63 cells after 24 hours compared to the controls

Many studies (Subramanian and Lin 2005; Wan et al. 2005) have demonstrated that the surface properties (i.e. surface texture and pore size) have great impact on cell adhesion, survival and proliferation. The MG63 osteosarcorma cells were used to evaluate the cell responses on the PLGA honeycomb films. In order to eliminate the influence of pore size, the honeycomb films were fabricated under the following conditions: PLGA50:50/$CHCl_3$ (1 g/l), PLGA75:25/$CHCl_3$ (1 g/l) and PLGA85:15/THF (20 g/l) at 26°C and 90% relative humidity. The resulting films had an average pore diameter of 4~5 μm, and were selected for cell viability tests. The studies were performed at 1, 3 and 7 days. The number of viable cells on the control (TCPS) was considered as 100% and cell viability on the honeycomb PLGA films were presented relative to the controls. In Figure 4, the honeycomb PLGA films for all testing points showed a percentage of viability above 100%, which indicated cells were all viable comparing to the control. After 1 day post-seeding, viable cells on all the PLGA groups were significantly higher than the TCPS groups (p<0.05) hence demonstrating that PLGA films with honeycomb structure improve early cell attachment most likely due to their

uniform porous structure, which was also observed by Beattie and co-workers (Beattie *et al.* 2006). After 3 and 7 days culture, the mean number of viable MG63 cells on all the PLGA films were still slightly higher than the TCPS groups, but no statistically significant differences were observed (p>0.05). This may be due to the cells having reached confluence after 3 days and starting to stack up on each other thus any substrate surface properties were negated in favour of cell-cell attachment. Also of note is that there was no significant difference (p>0.05) for cell number on PLGA 50:50, PLGA75:25 and PLGA85:15 over the 7 day culture. The fabrication conditions were chosen so that the honeycomb films had similar morphology. Therefore the only difference being the monomer ratio, these results indicate that cell attachment and proliferation over a 7 day period are not affected by the composition of PLGA films. Since the TCPS are smooth surfaces and the honeycomb films are not it can be inferred that surface structure is key to cell culture at least in the seeding and initial culture stages and is more important than the substrate chemical composition, when serum is used in the media.

MG63 filopodia 'hook' onto the pore edge

MG63 cells are anchorage-dependent and require attachment to a substrate for proliferation and normal function; adhesion is therefore a critical step that precedes these events. In this study, MG63 cells were used to investigate the cell responses to honeycomb structured topography and different compositions. The MG63 cell population was almost confluent and covered the surface of honeycomb structured films after 3 day (Figure 5); some cells stacked on top of other cells after this time (Figure 5a & b).

Some common morphological features were observed. It could be seen that cells interacted with all substrates; they were flattened, spread and presented cytoplasmatic prolongations on the surface of honeycomb films (Figure 5d, e & f). Protrusion of the leading edge of the cells on PLGA85:15 and PLGA75:25 were seen over a pore with approximately 5 μm diameter (Figure 5e & f). The filopodia of the leading edge were arranged radially and hooked inside of the pores, which act as anchorage points when cells exert physical forces for the movement (Figure 5e & f). The ability for cells to 'hook' onto the pores may be the reason for higher cell numbers seen on initial attachment

after 1 day. The cells cultured on the honeycomb PLGA films showed abundant microvilli on their surface which were indicative of cell activation (Xynos *et al.* 2000).

Conclusion

We have shown that a surfactant-free water droplet templating technique can be used to fabricate honeycomb PLGA films, and that choice of solvent, polymer concentration and humidity are all important factors in controlling the porous structure of the film. In order to obtain PLGA honeycomb films, polymer with a higher polylactide content (i.e. PLGA85:15) was more selective to THF as solvent. With decreasing polylactide content in polymer (i.e. PLGA75:25 and PLGA50:50), it became more selective to $CHCl_3$ as the solvent. Cell attachment and proliferation over 7 days was not affected by polymer composition, however the honeycomb films showed significantly higher cell numbers after 1 day. MG63 cells were able to hook onto the walls of honeycomb films during the culture. This indicated that the honeycomb structure has a favourable effect on the cell-biomaterial interaction; this evidence supports the theory that a structured surface improves cell attachment compared to smooth surfaces. At later time points of 3 and 7 days the cell number was comparable for all substrates.

The sensitivity of the surfactant-free water templating fabrication method has proven to be an ideal model system to investigate the factors affecting PLGA scaffold fabrication. It can be concluded that the scaffold structure is sensitive to fabrication conditions. Of particular interest the fabrication process was sensitive to PLGA composition, but cell attachment and proliferation was not. Therefore the PLGA composition can be selected to obtain the desired chemical, physical and mechanical properties of the scaffold without having to account for the effect of PLGA composition on early-stage cell attachment and proliferation.

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Figure Legends

Fig. 1. Surface structure of PLGA50:50, PLGA75:25 and PLGA85:15 films were created with different solvents ($CHCl_3$, ethyl acetate and THF) at a fixed polymer concentration. Honeycomb pattern formation was affected by solvent/water compatibility and rate of evaporation. SEM images showed: PLGA50:50 (a-c), PLGA75:25 (d-f) and PLGA85:15 (g-i) films prepared from $CHCl_3$ (left column), ethyl acetate (middle column) and THF (right column). Other conditions: Polymer concentration: 1 g/l, temperature: 26°C, relative humidity: 90%, spreading volume: 200 μl. Scale bar: 10 μm.

Fig. 2. The effect of polymer concentration on PLGA film structure, demonstrated with PLGA50:50 and PLGA75:25 in $CHCl_3$, and PLGA85:15 in THF. Increasing the polymer concentration changed the viscosity and surface tension of the polymer solution. The optimum concentration will be different for every casting solution. For PLGA50:50 and PLGA75:25 in $CHCl_3$, between 1g/l and 3 g/l gave the optimum honeycomb structure with the narrowest range of pore sizes and most regular pore structure. For PLGA85:15 in THF, 20 g/l gave the optimum honeycomb structure. SEM images of PLGA50:50 (a-e) and PLGA75:25 (f-j) films prepared from $CHCl_3$ with different concentrations: (a & f) 0.5 g/l, (b & g) 1 g/l, (c & h) 3 g/l, (d & i) 5 g/l, (e & j) 10 g/l; PLG85:15 (k-o) films prepared from THF with different concentrations: (k) 1 g/l (l) 10 g/l (m) 20 g/l (n) 25 g/l (o) 30 g/l; Other conditions: Temperature: 26°C, relative humidity: 90%, spreading volume: 200 μl. Scale bar: 10 μm.

Fig. 3. Effect of humidity on honeycomb structure. It was observed that at low humidity (<40%), no pores can be formed; however, pore sized increased with increasing of humidity. SEM images of PLGA50:50/$CHCl_3$ (a-c), PLGA75:25/$CHCl_3$ (d-f) and PLGA85:15/THF (g-i) films. Humidity values: 50% (left column), 70% (middle column) and 90% (right column). Other conditions: Temperature: 26°C, spreading volume: 200 μl. Scale bar: 10 μm.

Fig. 4. Viable MG63 cells on PLGA honeycomb films over 7 days culture. The viability of MG63 cells on the PLGA50:50, PLGA75:25, PLGA85:15, and tissue culture polystyrene (TCPS) were determined by XTT assay at 1, 3, and 7 days. Cells seeded on the TCPS without honeycomb PLGA films was used as a control and the viable cells on the TCPS was considered as 100% (average absorbance readings for the viable cells on the TCPS at day 1, 3 and 7 were 0.12, 0.21 and 0.48, respectively) . The results were expressed as mean ± s.d. for n=6. *and **, indicates significantly different from the control (p<0.05, p<0.001).

Fig. 5. Cell morphology on PLGA films after 3 days. (a, d) PLGA50:50 (b, e) PLGA75:25 (c, f) PLGA85:15. Top row shows cell distribution; Bottom row shows details of cell morphology. The arrow points to an example of a cell that has 'hooked' onto a pore. Scale bar: 10 μm.

Fig 1

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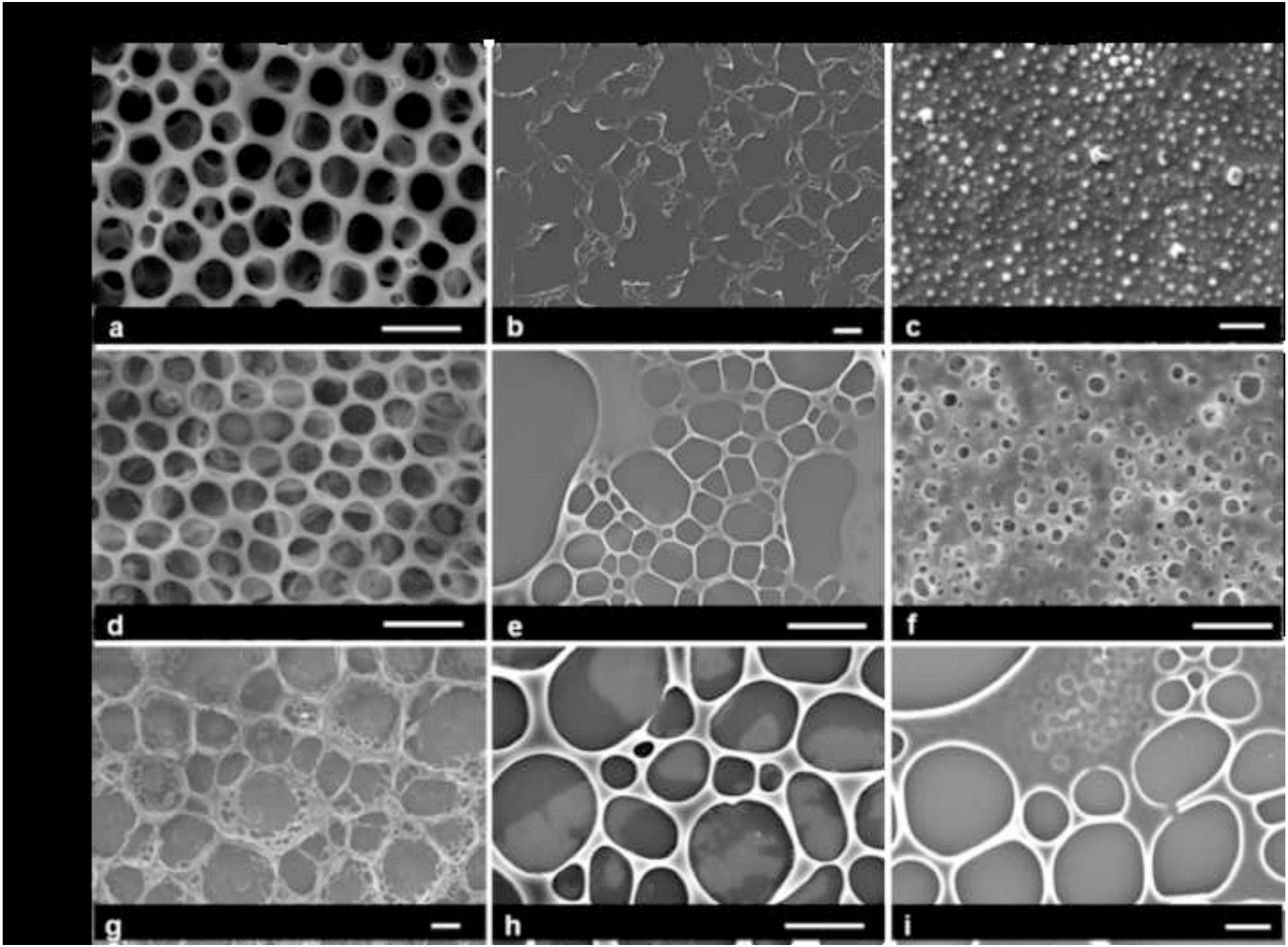

Fig 2

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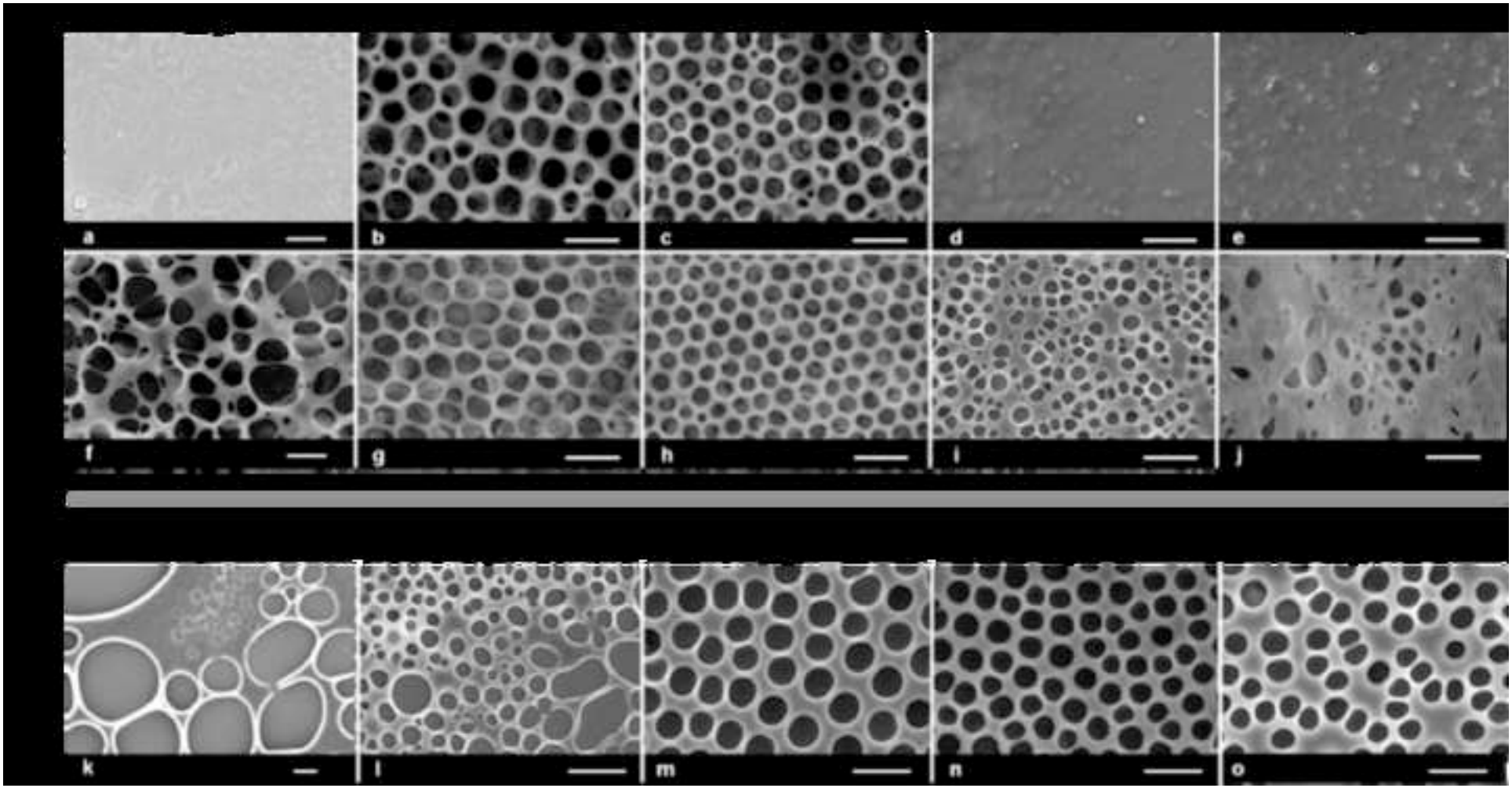

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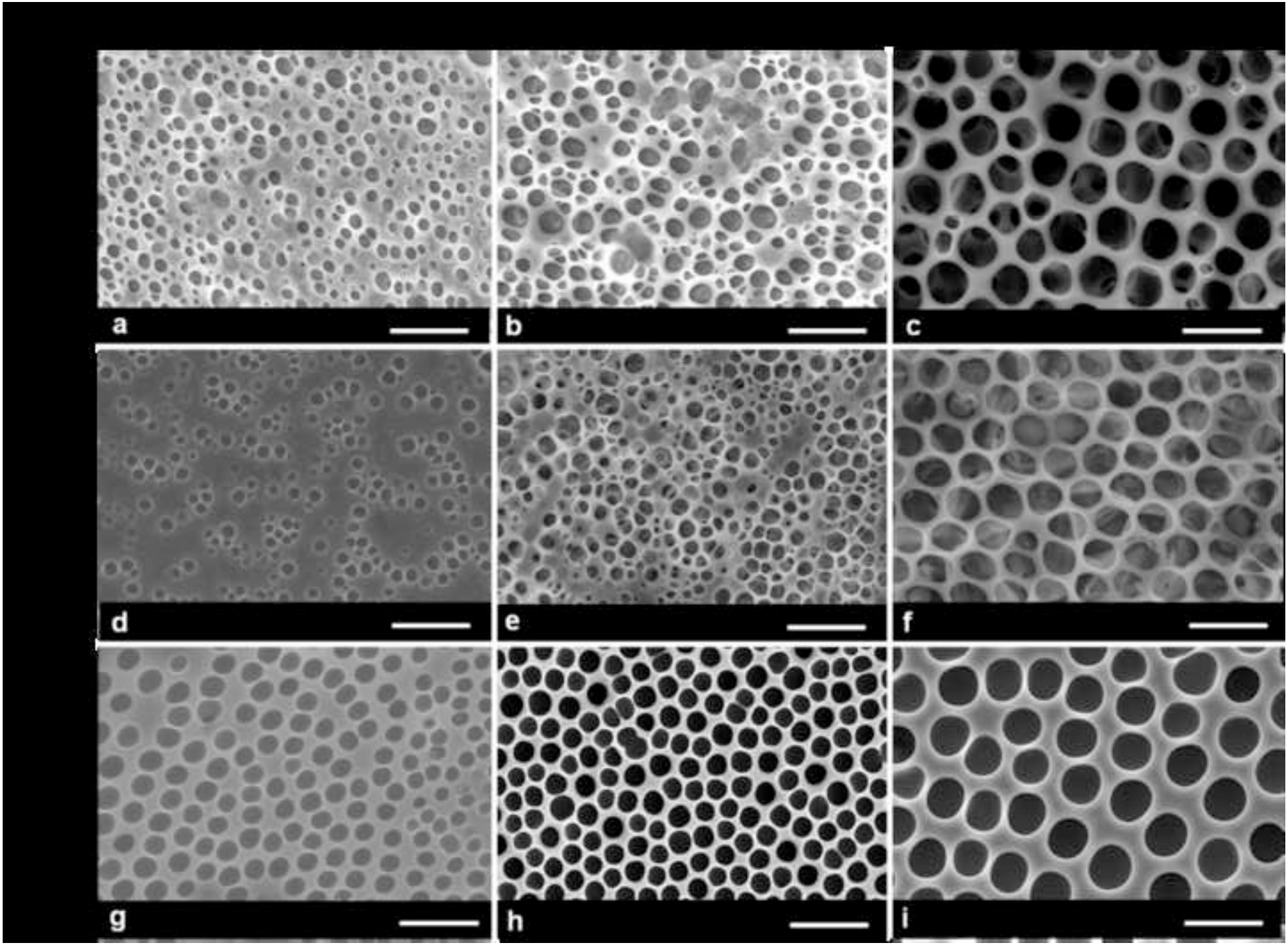

Fig 5

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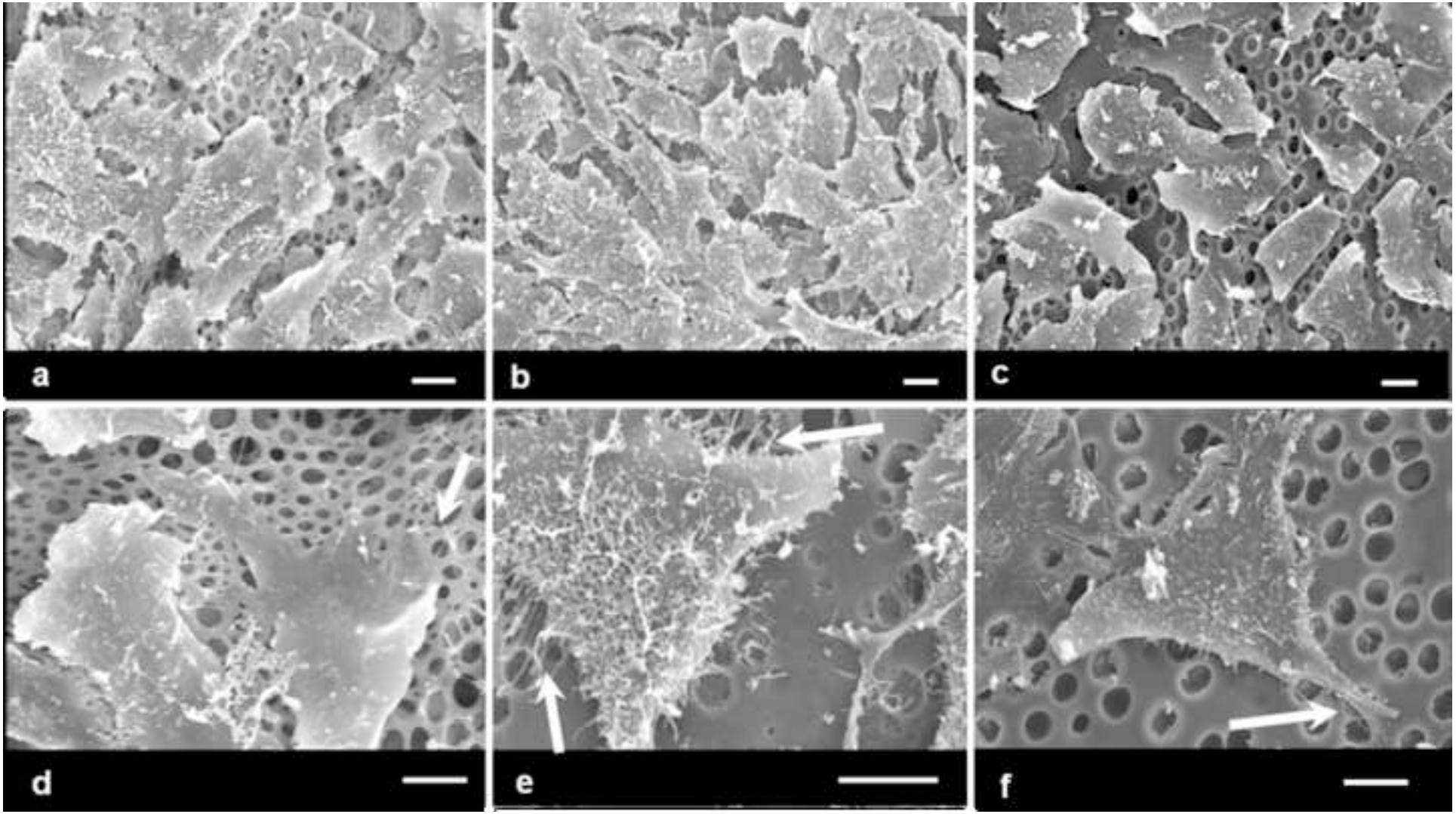

Fig 4

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