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Zirconia microbial hollow fibre bioreactor for *Escherichia coli* culture

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Abstract

Porous ceramic hollow fibre membranes can be used as bioreactors for microbe immobilization and growth. In this work, zirconia ceramic hollow fibre membranes were synthesised from 80 nm zirconia particles using a combined phase inversion and sintering technique The resulting membranes were characterised using SEM and XRD and tested for *Escherichia coli* immobilization. The membrane structural evolution with controlled *E. coli* growth was investigated. The well-maintained *E. coli* growth clearly shows that not only the hollow fibre lumen but also these micro-channels inside the fibre wall can be used as micro-bioreactors. Due to the unique structure properties, the prepared ZrO_2 hollow fibre membranes can find wide applications in microbe immobilization system.

Keywords: Hollow fibre; Zirconia; *E. coli*; Bio-ceramic membrane; Macro-encapsulation; Protein delivery

1. Introduction

Gram-negative *Escherichia coli* (*E. coli*) is one of the most commonly used prokaryotic hosts for recombinant DNA due to its capability for rapid growth, well documented genetic background and high yield of recombinant proteins [1]. The culture of genetically modified *E. coli* has also been actively studied for the production of heterologous proteins, microbial electricity and biomass energy [2–4]. There are several methods to cultivate *E. coli* in bulk. Conventional suspension systems generally consist of chemostat or other suspended growth-based biotreatment, but require subsequent separation steps. Alternatively, to increase cell reutilization and eliminate the need for expensive product recovery and purification processes a cell immobilization method is preferred. By avoiding the interference caused by fluid motion, this method provides an improved environment for maintained high cell activity. Cell immobilization can occur in two ways: surface immobilization or entrapment within specially designed microspaces. These immobilized microbial cells can be used extensively in both industrial applications and various scientific studies [5].

Among the different technologies for cell entrapping, membrane bioreactors have generated particular interest [6–11]. The membranes can be designed into a number of different geometries including flat plates, tubular or annular configurations [12]. Compared to other geometries, hollow fibre membranes provide many advantages including the highest surface area to volume ratios, the ability to act as a barrier against particle contamination and their high efficiency at retaining targeted microorganisms with reduced levels of resistance or by-product generation [13]. Bio-membrane reactors have been reported to provide a 40-fold increase in ethanol production by *Saccharomyces cerevisiae* (budding yeast) in comparison with batch operation [14].

In biological applications, mechanical stress from continued growth of bacteria and thermal stress caused during the fermentation process are usually encountered. Traditional polymeric hollow fibres suffer from problems of low solvent resistance and membrane rupture/distortion due to their poor mechanical strength [15–18]. Ceramics are promising alternatives owing to their increased structural, thermal and chemical stability, providing a material with high mechanical strength, toughness and unlimited autoclavability.

Based on the polymer phase inversion technique, inorganic hollow fibre precursors containing inorganic powder and the organic binder (polymer) can be prepared from the inorganic–organic mixture at room temperature using less expensive

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extruding equipments [19]. Depending on the final application, these fibre precursors can be sintered into fully densified or porous structure by adjusting the sintering programme. Zirconia hollow fibres have recently been developed with excellent mechanical strength reducing the problems of membrane rupture in cell immunoisolation [20–22]. However, different cell system poses different requirements on the membrane being employed as cell size and shapes are quite varied depending on the species.

In this work, we report the development of zirconia ceramic hollow fibres with unique properties to be applied as immobilized microbial cell reactors. The microstructure evolution of hollow fibre membrane bioreactor with controlled microbe (*E. coli*) growth was carefully investigated. This work is the preliminary results of our project with the ultimate goal of realizing protein or biofuel production from microbes in ceramic hollow fibre membrane reactors.

2. Experimental

2.1. Chemicals

Y_2O_3 (3 mol%) partially stabilized ZrO_2 powder with particle size of 80 nm [Shandong, China], N-methyl-2-pyrrolidone (NMP) [Synthesis Grade, Merck] and polyethersulfone (PESf) [Radel A-300, Ameco Performance, USA] were used as the raw materials to prepare the green hollow fibres. Untreated tap water and distilled water were used as the internal and external coagulants, respectively. Standard formulations of Bacto™ Tryptic Soy Broth (DIFCO, 17.0 g/l pancreatic digest of casein, 3.0 g/l enzymatic digest of soybean meal, 5.0 g/l sodium chloride, 2.5 g/l dipotassium phosphate, 2.5 g/l dextrose), both in analytical and biochemical grade, were used as the culture media and cell system, respectively. *E. coli* (ATCC25922) was stored at −80 °C after growth and subsequently transferred to fresh TSB before preparation of inoculum.

2.2. Preparation of ZrO_2 hollow fibre membranes

The ZrO_2–organic mixture was produced using 40% (wt) NMP, 10% PESf and 50% ZrO_2 and was thoroughly stirred to ensure uniform distribution of the ZrO_2 particles. The ceramic hollow fibre precursor containing ZrO_2 and PESf was extruded through a tube-in-orifice spinneret using a procedure described elsewhere [21]. The dried hollow fibre precursors were subsequently sintered at 1200 and 1400 °C for 10 h, respectively, in air atmosphere to form porous ZrO_2 ceramic hollow fibre membranes with respective bending strength of 49 and 220 MPa [21].

2.3. Bioreactor experiment

Short hollow fibres with length of 20 mm were sterilized at 120 °C and sealed on one end using zinc phosphate cement. The sterilized hollow fibres were immersed inside the autoclaved distilled water and Tryptic Soy Broth (TSB) both for 1 day. Ten microlitre cultures (produced fresh overnight) of *E. coli* were inoculated into 10 ml TSB and cultured until reaching mid-logarithmic-phase, ready for the hollow fibre encapsulation test. 100 μL *E. coli* (with approximately 0.1 optical density, reading at 600 nm) was injected inside the hollow fibre lumen and sealed on one side using zinc phosphate cement. ZrO_2 hollow fibres with embedded *E. coli* were then vertically suspended inside the soy broth with the sealed end just above the surface. Finally, the fibre sample was incubated at 37 °C with shaking (100 rpm) for 2, 6 and 16 h. *E. coli* viability and population inside the hollow fibres was carefully observed at various culture times.

2.4. Characterisation

Structures of ZrO_2 hollow fibre structures were examined using a scanning electron microscope (SEM, JEOL JSM-7400F and LEO 1550 VP field emission). After *E. coli* growth, the cells and hollow fibres were washed with 1× PBS three times then fixed in Formalin solution (10% neutral buffered, contains formaldehyde 4% w/v, Sigma–Aldrich) overnight. They were further washed with distilled water before being dehydrated using a series of ethanol washes and dried in a critical point dryer (Autosamdri-815, Tousimis Research Corporation, USA) and mounted onto aluminum stubs. The samples were coated with platinum prior to SEM observation.

3. Results and discussion

3.1. Preparation of zirconia ceramic hollow fibre membranes

Preparation of the appropriate hollow fibre precursor is the prerequisite for the successful application of this technology. Our experience indicates that the weight ratio of the inorganic particles to polymer for the fibre precursor should be arranged from 3 to 12 depending on the particle size and individual material density. Usually, the fibre precursor is more easily extruded using inorganic particles of higher density and larger particle sizes (0.1–50 μm) due to the improved flow characteristics. It is extremely difficult to prepare fibre precursors using pure nano-sized particles as the starting powder. For example, based on an alumina particle size of 10 nm, the hollow fibre precursor could not be made as it was impossible to achieve a homogenous alumina/polymer solution. This was in part due to the increase in slurry viscosity at above 10% (by weight). By contrast, the homogenous zirconia slurry appropriate for spinning can be prepared as zirconia has a higher density (6.0 g/cm^3) and a larger particle size (80 nm as observed from SEM in Fig. 1a) compared to that of alumina (density of 4.0 g/cm^3 particle size of 10 nm). Inorganic nano-particles with a particle size less of than 10 nm can be applied for many other advanced applications. As such nano-particles cannot be used in the fields of green ceramic extruding or casting because of its low particle loading density unless the addition of these nano-particles is limited to a few percent only.

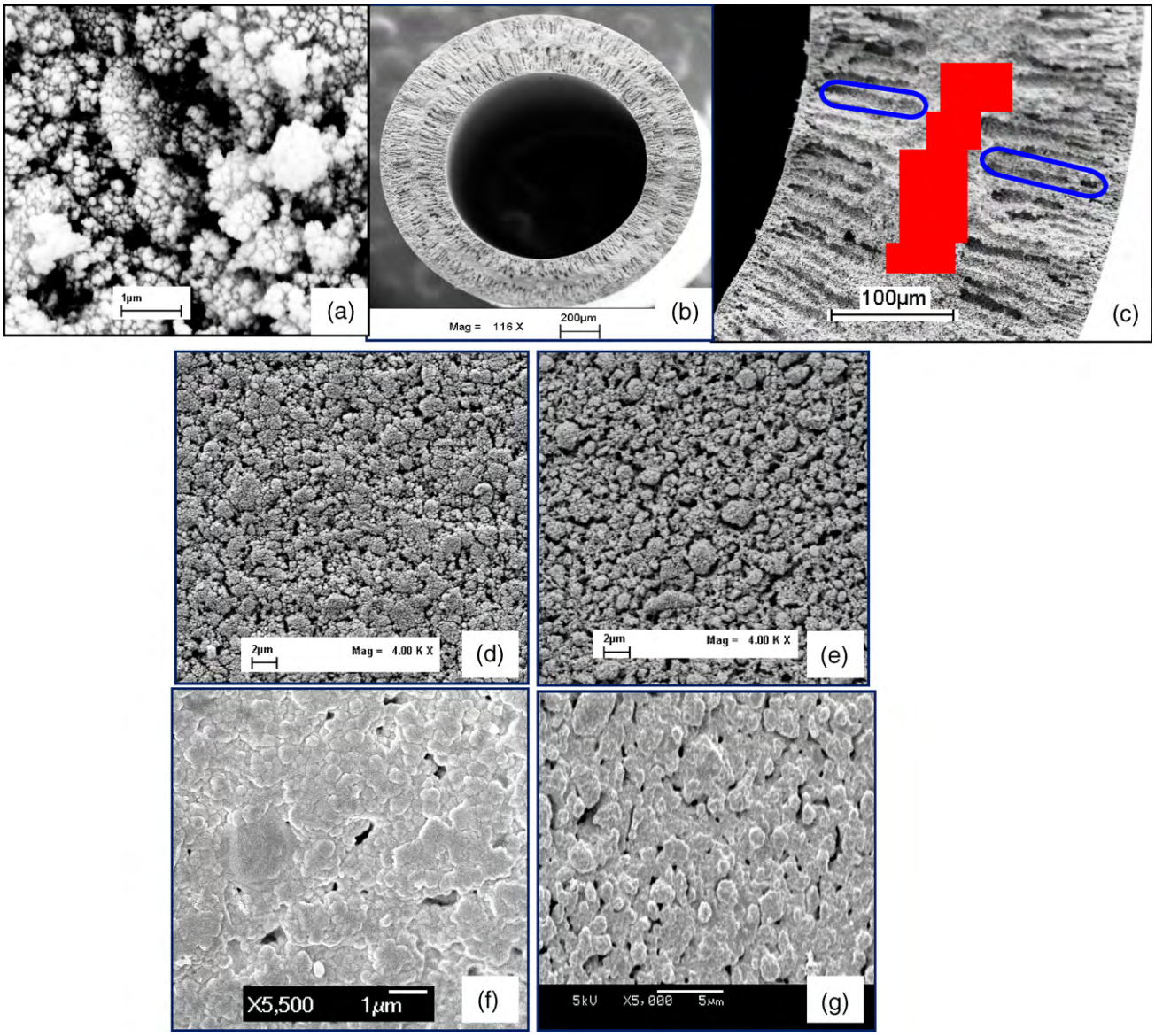

Fig. 1. SEM images of ZrO_2 particles used for hollow fibre material (a) and hollow fibre sintered at 1200 °C (b–e) or 1400 °C (f and g) for 10 h (b: cross section; c: magnified cross section; d, f: inner surface; e, g: outside surface).

The porous zirconia ceramic hollow fibre membrane with certain mechanical strength was attained by sintering at 1200 and 1400 °C for 10 h and the microstructure was observed from SEM images as shown in Fig. 1. The fibre o.d. and i.d. (Fig. 1b and c) are 1.5 and 1.0 mm, respectively. Fig. 1d–g show the effects of sintering temperature on the surface microstructure. When comparing these, it can be seen that sintering the ZrO_2 hollow fibre at a lower temperature resulted in a porous structure with higher porosity and bigger pore size but suffered from lower mechanical strength. Further to this it was found that fibres sintered at temperatures lower than 1100 °C showed such low mechanical strength that they were too weak to be routinely handled. On the other hand, when sintering was carried out at temperatures higher than 1450 °C (i.e., 1500 °C), the robust fibres would be too densified to be used as porous through structure. In this research, ZrO_2 hollow fibres to be used as bio-membrane reactors to culture *E. coli* were sintered at 1200 °C with a bending strength of 49 MPa [21]. Pores on the inner fibre surface (Fig. 1d) had a dimension of up to 1 μm width and 3 μm length particularly in the areas of larger particle agglomerates. By contrast, the outside surface (Fig. 1e) exhibited less porosity with pores predominantly smaller than 1 μm. The reason for this phenomenon has been described elsewhere [21]. Fig. 2 is the XRD patterns of the original ZrO_2 powder as the starting material and the prepared hollow fibre membranes. The starting powder consisted of both monoclinic and cubic fluorite crystalline phases. After sintering, the cubic phase predominates the XRD pattern of the prepared zirconia hollow fibres (Fig. 2b), which is a more favorable crystalline phase among the zirconia polymorph. Water contact-angle measurements confirm the good hydrophilic property of both surfaces.

Compared to the traditional flat and tubular membranes, the developed ZrO_2 hollow fibre membranes have the advantage of higher surface area/volume ratio. For example, given that the

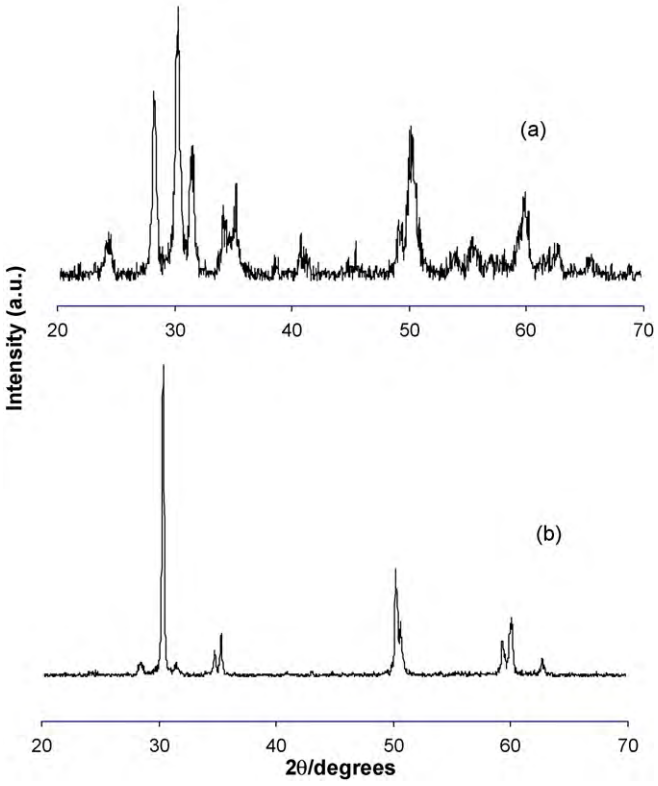

Fig. 2. XRD patterns of the ZrO_2 power used for membrane material (a) and the crashed ZrO_2 ceramic hollow fibres (b).

both surfaces of the fibre can be used, the fibre from Fig. 1b has a surface area (including the inner and the outside surface area) per membrane volume up to 4400 m^2/m^3. The micrograph of Fig. 1c illustrates the asymmetric structure of the prepared ZrO_2 membranes, which is a general characteristic of these hollow fibres prepared by the polymer phase inversion method. As seen in Fig. 1c, short finger-like channels (marked with blue shapes) were present near the outer and inner walls of the fibres while more densified sponge-like structures (marked with red rectangles) were located in the centre of the fibre. These short finger-like channels had a diameter of 5–10 μm, length of 70–100 μm and wall thickness of 8 μm, which were observed in the SEM image of Fig. 1c with higher magnification. For pure polymeric membranes, these finger-channels are often criticized because of their poor mechanical stability [23]. However, for ceramic membranes (particularly ZrO_2) which already possess high mechanical properties such channel structure does not pose any problems in application. In addition, the asymmetric structure offers more advantages for ZrO_2 hollow fibres in many biological applications as these additional microspaces in the finger-channels can be employed for cell or microbe encapsulation. In this case, theoretical calculation shows the overall surface area (two surfaces of the hollow fibres plus the inner surface of these finger-channels) per membrane volume will be further increased by a factor up to 20 since the surfaces of these micro finger-channels can also be counted for application. This highly improves the surface area/volume ratio of these hollow fibre membranes.

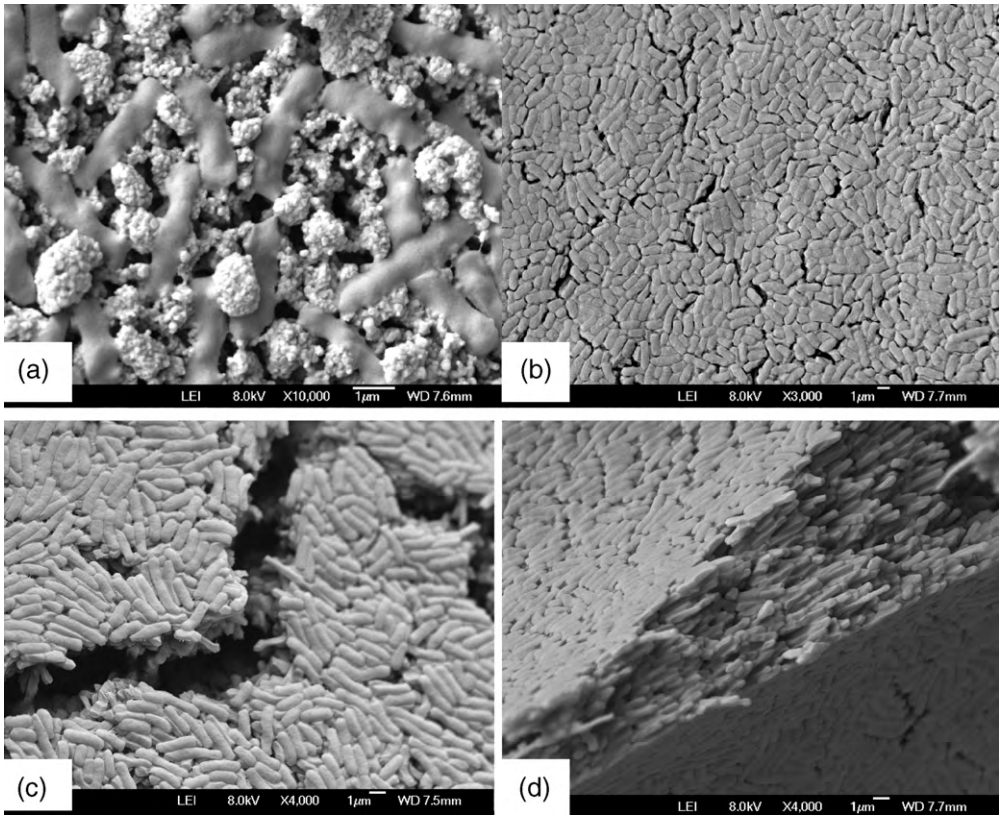

Fig. 3. SEM images of *E. colis* attached to the inside surface of the Zirconia Hollow Fibres at various culture times (a: 2 h; b: 6 h; c and d: 16 h).

3.2. Culture of *E. coli* in zirconia microbial hollow fibre bioreactor

E. coli were encapsulated inside the hollow fibre lumen and examined at increasing culture times to study the surface morphological change using SEM (Fig. 3). After culturing for 2 h, *E. coli* were loosely scattered. However, with the increased culture time the number of *E. coli* began to increase significantly. After growing for 6 h, *E. coli* could be seen to populate the entire inner surface of the ZrO_2 hollow fibre (Fig. 3b). This increased further with extended culturing as *E. coli* cultured for 16 h began to show multilayered growth (Fig. 3c and d). This multilayer adsorption highlights the ability of fibre lumen to store a higher density of *E. coli* within the growth liquid media. *E. coli*' escape from the fibre lumen surface to the outside nutrition environment was tested in this process through observing the turbidity of the culture solution. In the case that *E. coli* got into the culture solution, it became noticeably cloudy due to the fast proliferation rate of the bacteria. However, for all these experiments the culture solution

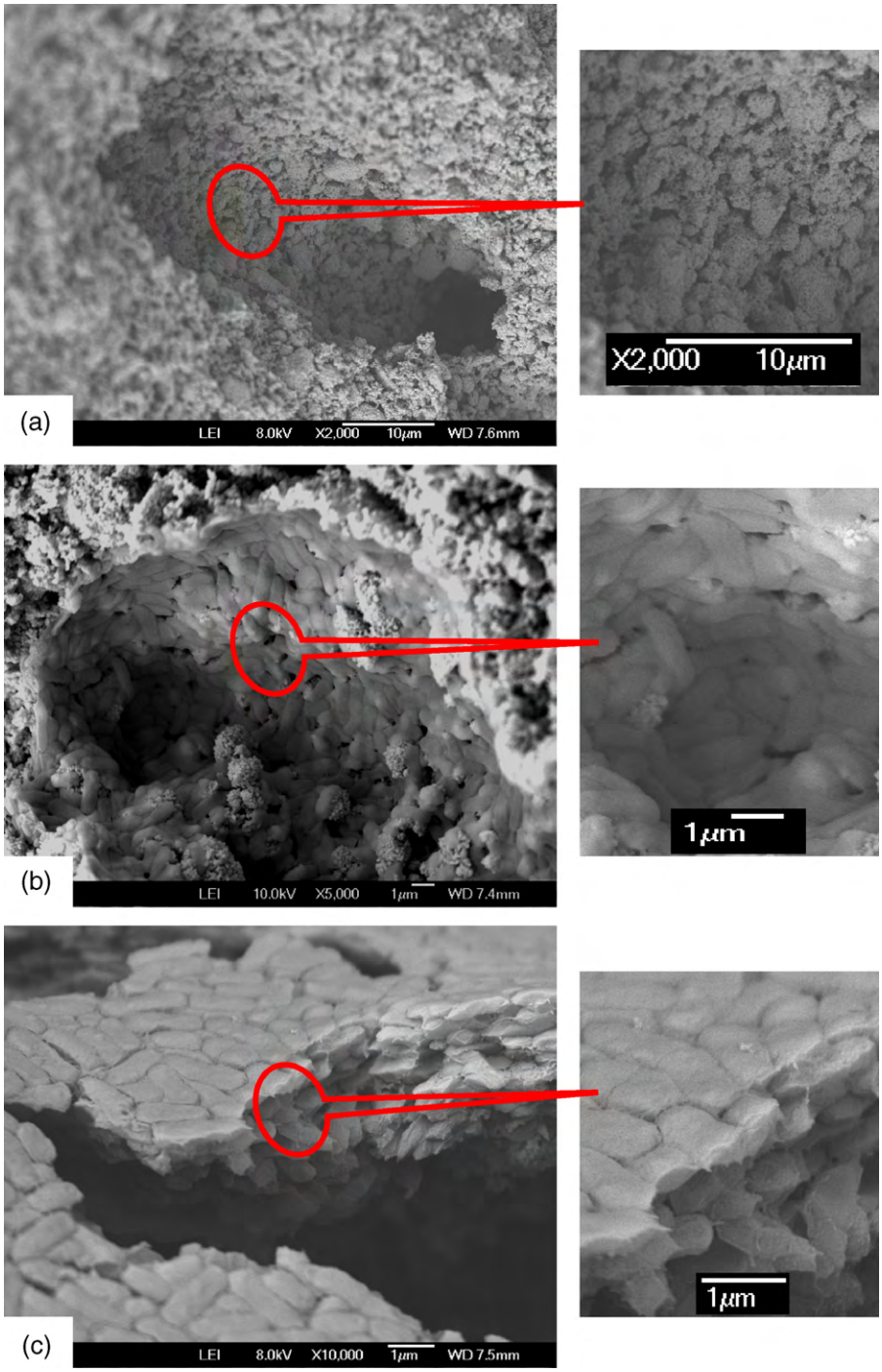

Fig. 4. SEM images of a typical finger-like micro-channels of Zirconia hollow fibre before and after *E. colis* growth (a: blank channel; b and c: *E. colis* inside the micro-channels with culture time of 6 and 16 h, respectively; SEM images in the right column are the magnified parts of the left column).

remained clear throughout the culture processes, indicating that there was no desorption of *E. coli* back into culture solution. The maintained growth of immobilized *E. coli* within the hollow fibre lumen indicated that critical nutrient ions or small bio-molecules like sodium chloride, dipotassium phosphate, casein, soybean protein and dextrose can freely permeate through the fibre wall from the outside surface. These nutrients can therefore access the fibre lumen to nourish the confined *E. coli* during the culturing process. However, *E. coli* cannot permeate from the encapsulated space to the outside due to their larger size. *E. coli* cells are typically rod-shaped, about 2 μm long and 0.5 μm in diameter. Successful isolation and growth of microbial cells is an absolute requirement for future genetically modified cell lines to be considered for many advanced applications. The use of polymeric materials in protein delivery has been often frustrated by protein denaturizing and fouling due to the hydrophobic nature of the membrane surface [24,25]. Cell growth in polymeric hollow fibre system was also investigated [26]. However, owing to the poor chemical resistance and mechanical stability, membrane ruptures and cell escape were often observed. By contrast, hydrophilic zirconia ceramic hollow fibre membranes with inherent higher mechanical strength show more promising future in these applications. As no *E. coli* growth was noticed in the culture solution during the whole culture process, it could be shown that the ZrO_2 membrane structural integrity was maintained and thus no cells could get through membrane.

Fig. 4 shows the morphological change of the finger-channels before and after the introduction of *E. coli*. As shown in Fig. 4a of a higher magnification SEM, the blank channel wall was composed of porous ZrO_2 particulates. After culturing for 6 h, *E. coli* covered the entire channel surface in a monolayer. As shown in Fig. 4c, with the extension of culturing to 16 h, *E. coli* proliferated, increasing the cell density in the finger-channel space. The *E. coli* seeds entered the micro-channels from the fibre lumen. After embedding inside the fibre lumen, it is possible for *E. coli* to find pathways through the larger pore pathways in the inner surface, to gain access to the finger-channels. Once *E. coli* penetrated through the inner surface, they were captured as seeds inside the finger-channels, where they have access to a ready source of nutrients and thus start to proliferate. Therefore, the successful application of these finger-like channels in the ceramic hollow fibre matrixes as the micro-bioreactors is quite dependent on the fibre inner surface properties and the cell size of the individual microbial system. For example, in this work, if the fibre is sintered at higher temperatures, both surfaces would undergo further densification with a decrease in porosity and pore size. In this case, it would become difficult for *E. coli* to enter the finger-channels and only the space from the fibre lumen could be used as a bioreactor. The added use of these finger-channels for bioreactors undoubtedly expands the application of these ZrO_2 hollow fibres. For instance, some microbes with smaller size in the initial growing stage can be seeded inside these finger-channels. With subsequent growth, these microbes could become immobilized or trapped inside the channels while the cell products (bio-molecules or proteins) can still be removed from the fibre. With the existence of two different channel layers separated by the central densified layer inside the hollow fibre structure, it is possible for two different microbial systems to be applied to the same ceramic hollow fibres. To our knowledge, this is the first report of micro-bioreactors using these finger-like micro-channels in the ceramic hollow fibre matrix.

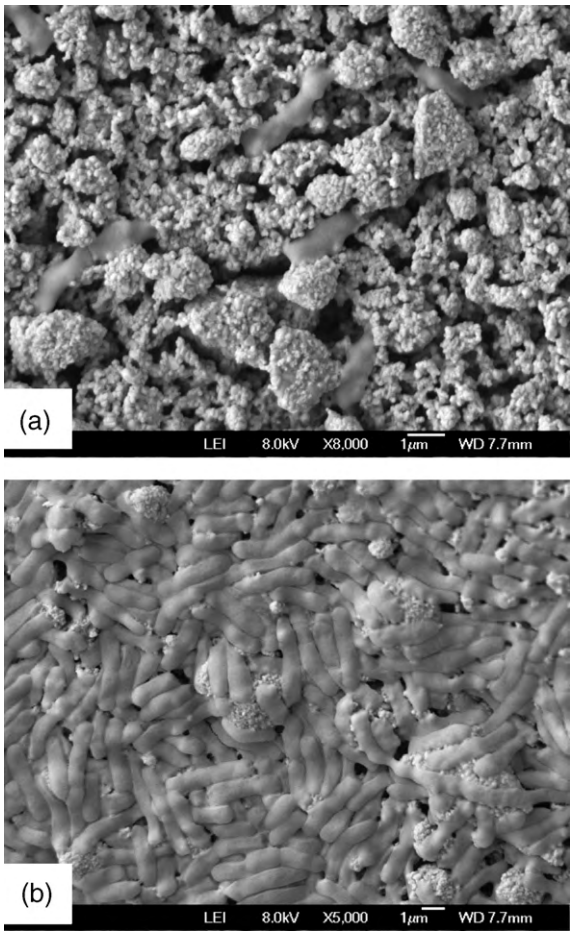

Fig. 5. SEM images of *E. colis* attached to the inside surface of the zirconia hollow fibres near the surface level of the culture solution (culture time: a, 2 h; b, 6 h).

Fig. 5 shows the *E. coli* distribution on the top area of the inner surface of the hollow fibres. In comparison to Fig. 3a and b and Fig. 4a and b, it is clear that *E. coli* were less populated on the top area than that of the bottom area. This implies that when the hollow fibre is vertically suspended in the nutrition solution, *E. coli* distribution along the lumen direction is not uniform, shown through a decrease in cell population density on the upper sides of the tube. It is possible that *E. coli* have the tendency to settle down to the fibre bottom because of the gravity effect. To get a uniform *E. coli* distribution, the ZrO_2 hollow fibre should be horizontally placed inside the broth.

4. Conclusion

Zirconia ceramic hollow fibre membranes were developed using room temperature extrusion from a polymer–zirconia mixture followed by subsequent sintering at 1200 °C for 10 h.

This fibre extrusion technology has special requirement for the particle loading density. Inorganic nano-particles with sizes less than 10 nm alone cannot be applied for this technology because of its low particle loading. In this work, the zirconia particles used for extrusion has a size around 80 nm. The hollow fibre membranes possess asymmetric structure with two finger-channel layers near the outside and inner surfaces and a more densified layer at the fibre wall centre. The membrane was tested as a bioreactor for *E. coli* culture. The maintained growth of the successfully encapsulated *E. coli* clearly shows that not only the hollow fibre lumen but also these micro finger-channels in the fibre matrix can be used as micro-bioreactors. By increasing the available spaces for cell encapsulation and improving the cell retention within the membrane structure, these hollow fibre membranes provide a unique opportunity that could expand the potential application of the resulted ZrO_2 hollow fibre membranes in microbe immobilization.

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