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SCHOLARENA

Journal of Nanoscience and Nanotechnology Applications

RESEARCH ARTICLE

ISSN: 2577-7920

Fabrication of Honeycomb Patterned Polyurethane Membranes for Biomedical Applications

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Citation: Velayudhan S, Prabha D Nair (2020) Fabrication of Honeycomb Patterned Polyurethane Membranes for Biomedical Applications. J Nanosci Nanotechnol Appl 4: 101

Article history: Received: 28 January 2020, **Accepted:** 09 June 2020, **Published:** 10 June 2020

Abstract

This paper reports the fabrication of self-organized honeycomb-patterned polyurethane (PU) membranes by water droplet templating method. Honeycomb-patterned, porous membranes with pore sizes ranging from 3-25 μm were obtained by controlled evaporation of PU solution in chloroform under humid atmosphere. The influence of polymer concentration and humidity on the pore size of the resulting membrane was investigated in detail. Hydrophilized membranes were also produced by casting a solution of PU blended with traces of polyvinylpyrrolidone (PVP). It was noted that addition of PVP did not disrupt the formation of ordered honeycomb patterns. Instead, PVP aided in the formation of honeycomb patterns, even at higher concentrations of PU solution, which otherwise was not feasible. Incubation of the hydrophilized honeycomb membranes with bovine serum albumin solution (BSA) over a period of 24h indicated that these membranes were less prone to fouling. Cell (MSC) attachment studies conducted on hydrophilized patterned membranes showed that lesser number of cells adhered to them when compared to their un-patterned counterpart.

Keywords: Breath Figure; Honeycomb; Polymer; Membranes

Introduction

Creation of well-defined, stable and uniform membranes has been a matter of great interest for many biomedical applications such as protein separation, blood filtration, and many implantable devices (such as bio-artificial pancreas, biosensor). Apart from meeting the important requirement of biocompatibility the membranes for these purposes must exhibit minimum fouling and structural stability under wide range of biological conditions. Traditionally, polymer membranes are produced by making use of the so-called phase inversion processes. Though simple and versatile, phase inversion technique mostly produces membrane with a wide variance in the distribution of the pore size. Such membranes potentially carry two disadvantages: (1) They do not permit precise separation of a substance and (2) are also at high risk of fouling. This is understood as a fast blocking of large pores, since a larger portion of the liquid passing through the membrane first passes through the large pores. In this scenario, the fabrication of isoporous membranes (i.e., membranes with low variance in the distribution of their pore size becomes) imperative combat the shortcomings of traditional phase inversion membranes.

Of the many methods employed for fabrication of isoporous polymer films, water droplet templating method otherwise known as breath figure (BF) technique has attracted much interest due to its simplicity and cost-effectiveness [1-3]. The BF technique employs solvent-evaporative cooling on the surface of a solution under humid conditions on which water vapor is condensed and water droplets are formed at the interface of the solution and water vapor [4]. The water droplets then interact with one another and are finally ordered in a hexagonal lattice. After complete evaporation of the solvent and water, traces of water droplets remain in the polymer film leading to the formation of pores with a honeycomb structure. Thus, the key step to preparing ordered porous structure by BF on a polymer film involves stabilizing condensed water droplets on the volatile solution.

In order to realize this condition, polymers with special architectures (viz., star polystyrene SPS and rod-coil block PS dendrimers, amphiphilic polymer) were synthesized and BF films fabricated [1,5-8]. However, recent studies showed that a variety of polymers such as linear conjugated polymers, polyimides, light emitting polymers, liquid-crystalline polymers, organo-metallic polymers and

degradable polymers could also produce BF templating under suitable conditions of solution viscosity, humidity and temperature [4,9,10]. In interest of the vast potential the BF templating offers, researchers have attempted applying this technique on many biocompatible and biodegradable polymer systems [11-14]. While some of the polymer systems required the use of biocompatible surfactants to form honeycomb structures [15], others form honey-comb patterned films without the aid of any surfactants [16].

Polyurethanes (PU) have found a niche in biomedical applications because of their interesting mechanical and biological properties. Polyurethanes are perhaps the most bio- and blood-compatible materials known today and has played a major role in the development of many medical devices ranging from catheters to total artificial heart. PU have also found applications as membranes for immunoisolation [17] and also as protective membranes for implantable biosensors [18]. Lately, biodegradable PU have been proposed to be used as porous scaffolds for cancellous bone graft substitutes and microporous membranes for the treatment of articular cartilage defects [19,20]. In regard to the application of microporous membranes to cell culture substrates or as scaffolds for cells, it is recognized that cell adhesion, migration and proliferation are considerably influenced by the surface structures of materials [21]. Since the micro-size structure on the material surface is important to control cell behavior, much attention has been directed toward technology for fabricating the material surface. Some authors have reported that the honeycomb-patterned micro-size structure on a polymer surface can control cell activity arbitrarily [22-24].

This study addresses for the design and fabrication of a well-ordered microporous membranes from polyurethane in chloroform via the BF method. Honeycomb pattern membranes were fabricated both in the presence and absence of surfactant (Polyvinylpyrrolidone). The influence of polymer and surfactant concentration, and humidity of atmosphere on the membrane structure were investigated. The membranes were characterized for the diffusion and antifouling capabilities and also tested for their compatibility with MSC. With the vast potentials that this versatile polymer offers in the area of biomedical research, it has been envisaged that these well-ordered membranes could possibly serve as cell carrier for matrix-assisted implantations.

Materials and Methods

A commercial PU Chronothane was purchased from (AdvancSource biomaterials, USA) and dissolved in chloroform (SD Fine, India) at 25 °C to obtain polymer solutions of 1-5% (w/v) concentration and used within 18h to produce porous films. Polyvinylpyrolidone (PVP, Mw=30 K) procured from (Sigma, USA) was used as the surfactant and was added in various concentrations from 0.1% to 2 % by weight based on the weight of PU. All the materials/chemical regents were used as received without further purification. For the fabrication of honeycomb-patterned films glass substrates (0.5 x 1cm^2) were cleaned by detergent and acetone successively and air dried. Glass substrates were placed inside an acryl box where a hot water beaker controlled relative humidity (RH) determined by a hygrometer. Polymer (PU and PU/PVP) solutions were then dropped on the cleaned glass substrates and directly evaporated under humid condition.

The honeycomb-pattern was observed in SEM (JEOL Model JSM - 6390LV, Japan) and AFM (Agilent Technologies, USA). The fouling characteristics of the honeycomb membranes were evaluated using Bovine serum albumin (BSA) as a model protein [25]. The static BSA adsorption experiment was performed as follows: Drop cast membranes with approx. surface area 28cm^2 were incubated at 37 °C for 18 h in a 1 mg/mL BSA solution in PBS. At the end of 18 h the membranes were removed from the protein solution and incubated three times for 20 min in PBS buffer. The membranes were then immersed into glass tube containing 1 wt% aqueous solution of Triton X-100 and shaken for 60 min at room temperature to remove the adsorbed protein. The amount of protein adsorbed on the membrane surface was calculated from the concentration of protein in Triton using protein analysis kit (Micro BCA, protein reagent kit). Measurements of triplicate samples were performed and averaged.

Mesenchymal Stem Cells (MSC) were used to assess cell attachment and proliferation on the honeycomb-structured films. The animal experimental protocol was approved by the Institutional Animal Ethics Committee of Sree Chitra Tirunal Institute for Medical Sciences and Technology. Mesenchymal stem cells were isolated from bone marrow aspirates of New Zealand white rabbits which were 4-6 month old. Mononuclear cells were isolated by density gradient centrifugation using Histopaque 1.077 g/ml density, (Sigma). The cells were expanded on DMEM with high glucose (Gibco, India) containing 10 % foetal bovine serum, 10 U/mL penicillin and 100 µg/mL streptomycin (Gibco, India) at 37 °C in a humidified 5% CO_2/95% air incubator (Nuaire, USA). The cells were harvested by Trypsinization and cells of passage 4 were used for the experiments. Non-adherent cells were removed by changing the culture medium after 5 days of culture. The cells were harvested by Trypsinization and cells of passage 4 were used for the experiments. Thin membranes (ϕ = 10mm) were sterilized by submerging them in 70% ethanol for 30 min and washing twice with an excess of phosphate buffered saline (PBS). The cells were seeded onto the thin film-coated glass cover slips with an initial cell density of 12,000/cm^2 in 6-well culture dishes, and were maintained in a humidified environment at 37 °C and 5% CO_2. The media was changed after 6 h in and the adhesion and viability of cells were observed by Live-Dead assay kit (Molecular probes) and imaged using Fluorescent microscope (Leica, Germany).

Results and Discussion

Effect of Polymer Concentration

Initially films were prepared from PU by casting 100 µl of polymer solution on a cleaned glass substrate under humid environment at 25 °C. Figure 1 show micrographs of PU membranes produced by direct evaporation of PU solutions at 90% RH. It was noted

that regular pores with honeycomb like structure (BF patterns) were generated for PU solutions at lower concentrations. This indicates the capability of PU solution to stabilize condensed water droplets without coalescence though the polymer grade used does not have an associative functional group, amphiphilic moiety, and star-shaped molecular architecture, which are known to be essential for generating BF patterns. It has been reported by Srinivasarao and co-worker that certain homopolymers can stabilize water droplets in water-immiscible solution and form ordered architecture [26]. Furthermore, Peng, *et al.* [10] showed that well-ordered porous structures by the BF method could be prepared by evaporating polystyrene (PS) solution in toluene under humid conditions when the proper molecular weight (Mw) of PS was used. In yet another report, it was shown that the honeycomb-structured porous PLLA films could be fabricated by evaporating PLLA solution in THF directly under certain humidity without an addition of amphiphilic components [11].

For PU solution with higher polymer loading, i.e., 4% (w/v), BF patterns formed lacked lucidity (Figure 1c). The pore like impression formed on the surface appeared shallow and disordered (Figure 2a). Connected pores were formed on the surface of films when the concentration of PU solution increased to 5% (w/v) suggesting that a proper viscosity of PU in solution is important to generate well-ordered pores [10]. Breath-figure patterning is a water-assisted method, which utilizes the water droplets as templates condensed on the solution surface by quickly evaporating the solvent. The formation of ordered patterns with low concentrations of PU solutions contributed to the convection generated in the evaporating solution and the lateral capillary force between the adjacent droplets [11]. When the PU solution concentration increased to 4 and 5% (w/v) the solution viscosity increased, which in turn weakened the convection in the solution, thus hampering the ordered packing of droplets. Conversely, the increase of concentration also accelerates the phase-inversion process leaving the droplets with very less time to sink into the solution and pack orderly before coalescence. The disordered morphology was formed after the solvent and water droplets evaporated completely.

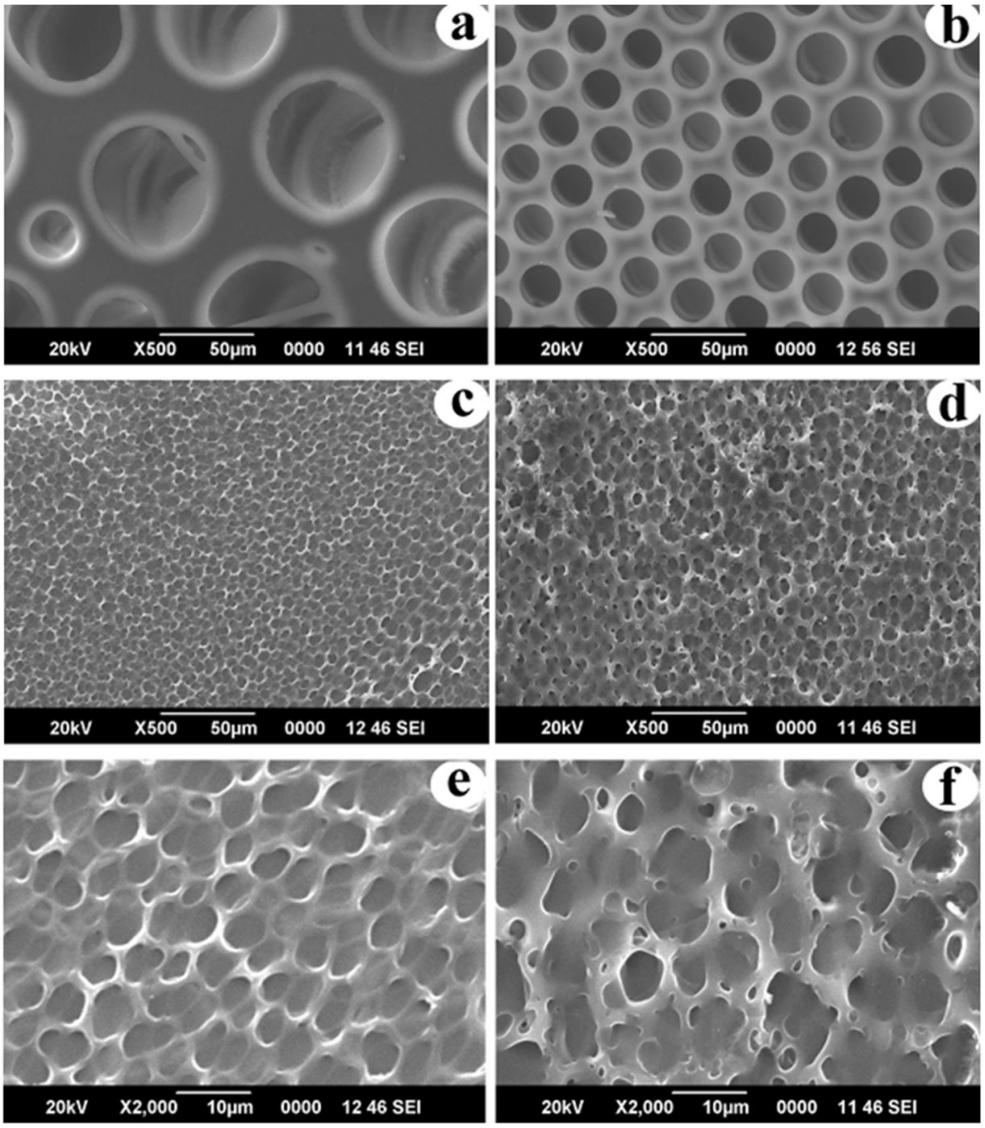

Figure 1: The micrographs of membrane surfaces of PU at 25 °C and 75% RH (a) 1%(w/v); (b) 2%(w/v); (c) 4%(w/v); (d) 5%(w/v); (e) and (f) are magnified images of (c) and (d), respectively

It was found that with the increase in polymer concentration, the pore size decreased from 70 μm (1% (w/v) to 7 μm (4 % (w/v) as the PU solution concentration increased from 1% to 4% (w/v). Theoretically, the solvent in a more concentrated solution experiences a lower vapor pressure. This reduces the evaporation rate of solvent and leads to a higher surface temperature. It has been reported that the growth rate of droplets is proportional to the temperature difference (ΔT) between the atmosphere and the solution surface which was described as follows:

$$dR/dt \sim \Delta T^{0.8}$$

Where the R was the radius of droplets. As per this equation, a higher surface temperature slowed the growth rate of droplets because of the small ΔT (the atmosphere temperature was kept at 25 °C in the process of film formation). Thus, a viscous polymer solution that results from the increased polymer concentration retards the growth of the water droplet [27]. Besides, a concentrated polymer solution would hasten phase-inversion (solidification) and curb the growth time of the droplets to result in membranes with the smaller pores. It should be noted that with the virgin polymer, spherical pores with typical aspect ratio of one were obtained.

Effect of Additive

In an attempt to produce honey-comb patterned films from concentrated PU solutions; it was blended with various quantities of PVP and drop-cast under humid environment. Table 1 show the capability of PVP to form the honeycomb patterned structure (25 °C; 75 RH) when blended with 4 (w/v) % of PU solution. As is evident from Table 1 the phenomena of honeycomb pattern formation were observed at PVP concentrations from 7.5 to 20% by weight based on the weight of PU.

PVP concentration (wt.%)	Honey-comb pattern	Pore diameter, μm
0 – 7.5	No uniform pattern	7.9± 0.3
7.5	Honey-comb pattern	6.5 ± 0.5
10	Honey-comb pattern	5 ± 0.5
20	Honey-comb pattern	4.5 ± 0.5
25	No uniform pattern	3.5 ± 0.5

Table 1: Effect of PVP concentration on the membrane pattern and pore diameter

Similar results were published by Vamvounis, *et al.* [8] who reported the fabrication of high quality hexagonally close packed membranes, from higher loadings of poly(9,9′-dihexylfluorene) (PDHF) by blending with various ratios of polystyrene-grafted silica nano-particles (Si-g-PS), which was otherwise possible only at low concentrations of virgin PDHF. It is known that the stabilization of droplets is an important element to format the films with the honeycomb structure. Hence for polymer systems those are incapable of stabilizing the water droplets are often "upgraded" by blending in suitable moieties that encourage the stabilization of water droplets. In this study, PVP was chosen to act as the surfactant that contributes to the stability of the water droplets by self-assembly to the interface between the polymer solution and the water droplets to help in the formation of honeycomb-patterned membranes. Besides being hydrophilic, PVP is biocompatible and blends well with PU in various ratios. It also dissolves in PU/chloroform solution easily.

The phenomena that the PU solution could form honeycomb structures at higher polymer concentration in the presence of PVP could be attributed to the change in surface tension of the solution. Addition of PVP to PU solution makes the PU/PVP system hydrophilic. The hydrophilic PVP trends to aggregate strongly on the surface of the solution and forms a thin film. This film reduces the surface tension of the solution and stabilizes water droplets in the solution to ensure uniformity of the water droplets [13].

The diameter of the honeycomb pore ranged from 6.5 to 3.5 μm when the concentration of PVP varied from 7.5 to 25wt%. When the PVP concentration was < 7.5 wt. % honeycomb-pattern was not observed. Instead, pore impressions of diameter 5.5 μm were seen scattered on the surface of the membranes. Similar was the case, when PVP concentration was > 20wt%; honeycomb pattern was not formed even when pores of diameter as small as 3.5 μm were produced. This phenomenon could be explained as follows.

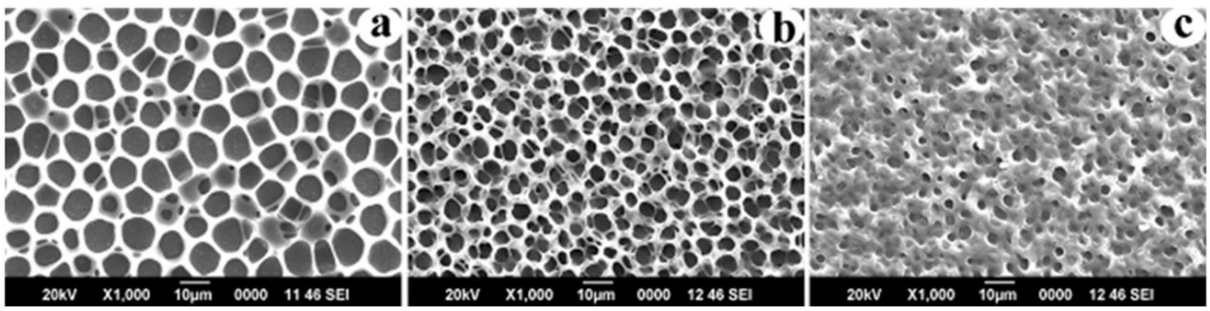

Figure 2: SEM images of porous membranes prepared using PU solution with different content of PVP: (a) 7.5, (b) 10, (c) 25 wt.%. Other conditions: concentration, 4% (w/v); temperature, 25 °C; RH, 75%; spreading volume, 100 μL

When the concentration of PVP was below 7.5 wt.%, the number of PVP molecules in the solution was low, which limited the number of PVP molecules that assembled on the surface. As a result a weak surface film was formed which could not totally prevent

the collision of water droplets resulting in the formation of disrupted honeycomb pattern. When the PVP concentration increased (i.e., > 7.5 wt.%), a greater number of PVP molecules assembled on the surface of the solution and formed a strong interfacial film, which could attract and stabilize the water droplets. The patterns were found to be more regular (Figure 2a & 2b). However, when the PVP concentration increased beyond 20 wt. % the structure gradually became disordered (Figure 2c). This could be because when the concentration of PVP solution was too high, many polymer molecules gathered on the surface of the solution, and formed a compact film, which led to high surface tension of the solution. As a result water droplets could not closely arrange on the surface of the solution and act as the template of the pattern, resulting in the formation of distorted pore structures [13,28].

This study utilized 7.5 wt. % of PVP to fabricate honeycomb patterned structure from PU at higher concentrations, which is slightly higher than the surfactant concentrations employed by other researchers to fabricate similar structures on polymers like PCL and PLA. For instance, Nishikawa required about 5wt% of surfactant concentration to fabricate honeycomb PLA [21] while Fukuhira, *et al.* [15] produced them by using just 0.2 wt% of the surfactant. A higher amount of PVP in PU is, however, advantageous as it induces hydrophilicity to the otherwise highly hydrophobic PU membrane. Besides, presence of PVP could also aid in reducing the membrane fouling and improves the blood compatibility.

Effect of Humidity

The BF method to fabricate well-ordered macroporous membranes utilizes the condensed mono-disperse water droplets as templates when the solvent of polymer solution is evaporated [29], therefore the surface morphology of the membranes is directly depended on the atmospheric humidity. In order to investigate this, the membranes were fabricated using PU solutions loaded with 7.5 wt.% PVP and the solvent evaporated directly at 25 °C at various humidity from 40 to 90%. The surface images of the membranes were shown in Figure 3.

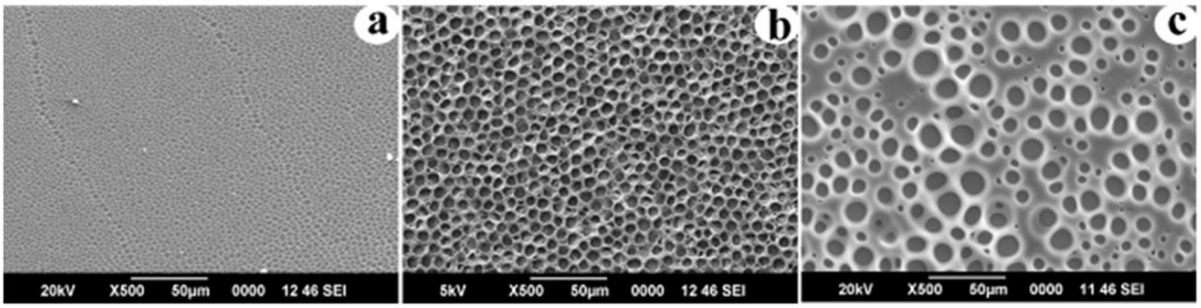

Figure 3: SEM images of porous membranes prepared using PU solution with 7.5 wt.% of PVP at different humidity conditions (a) 50%, (b) 75%, (c) 90 %. Other conditions: concentration, 4% (w/v); temperature, 25 °C; spreading volume, 150 µL

At 40% RH, smooth and transparent membrane was formed (Figure 3 not shown). Disordered pores began to be formed on the surface of the membrane with the increase in humidity; relatively regular pores were obtained at 50% RH (Figure 3a), and the membrane became opaque. The pores were found to be further organized at 75% RH (Figure 3b). However, when the humidity was increased further to 90%, the pores became irregular (Figure 3c). These results showed that well-ordered membranes were successfully fabricated by evaporating the polymer solution at 50–75% RH. Nonetheless, regularly ordered patterns were not produced at the other humidity. This phenomenon could be explained by taking into account the growth mechanism of the water droplets in a breath figure process.

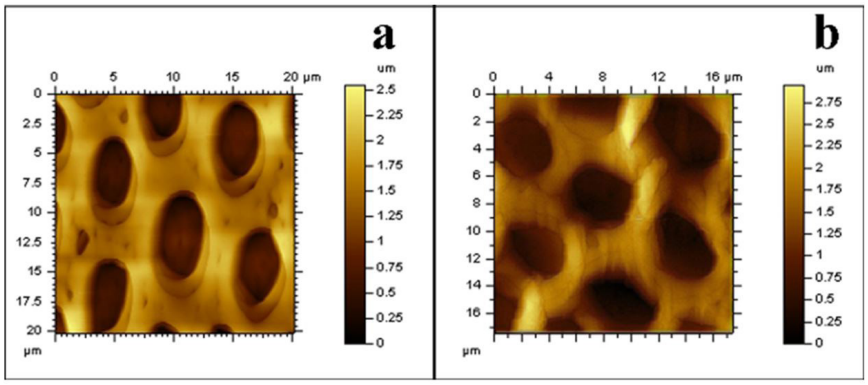

Figure 4: AFM images of porous membranes prepared using PU solution with 7.5 wt.% of PVP at different humidity conditions (a) 50 %, (b) 75%. Other conditions: concentration, 4% (w/v); temperature, 25 °C; spreading volume, 150 µL

At low humidity condition (40% RH) enough water droplets were not quickly formed to cover the whole surface of the solution, hence membranes with ordered patterns could not produced. Besides, at 40% RH, the viscosity of solution increased with the evaporation of solvent. Consequently, water droplets could not sink into the solution, which lead to the coalescence of some water droplets. When the RH increased to a much higher level, the solution surface gets covered with lots of condensed water droplets

which sink into the solution in a short period of time. The viscosity of solution also increased with the evaporation of the solvent which prevented the water droplets from diffusing and colliding [30]. When the solvent evaporated completely, ordered patterns were observed as shown in Figures 3a, 3b & 4. However, at 90% humidity, the water droplets were formed too quickly due to the high rate of nucleation, resulting in the coalescence, which resulted in the formation of pores with irregular pattern Figure 4.

The average pore diameters (Dn) of the membranes prepared at different RH were obtained by analyzing the SEM images using ImageJ Software and plotted in Figure 5. It is clear from the plot that the pore diameters were found to be affected by the RH; with larger pores produced at higher humidity levels. The apparent diameter (i.e., pore lateral dimension measured on the film surface) was Dn (9.09±0.05μm) for RH 90% while it was reduced to Dn (4±0.05μm) for RH 50%.

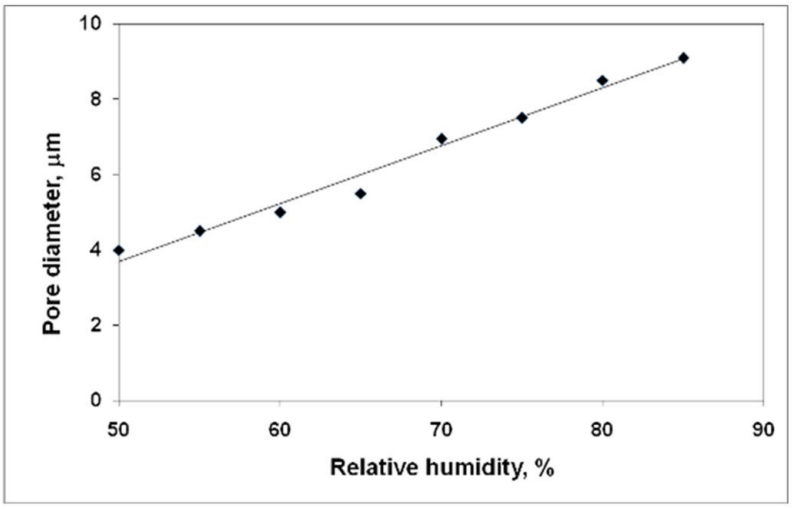

Figure 5: Dependence of membrane pore size on the relative humidity

Protein Adsorption and Cell Adhesion

The BSA adsorption experiment was performed to investigate the membrane antifouling property. Two membranes samples namely virgin PU and PU/PVP membrane loaded with 7.5 wt% PVP (designated as PU/PVP_7.5) were used for the protein adsorption studies and the results given in Figure 6. It can be seen that for BSA solution of 1mg/ml, the adsorption amount on PU/PVP_7.5 was 13±3μg/cm^2. This value was much smaller than virgin PU membrane, which was 33±4μg/cm^2. This low absorption of protein on PU/PVP membranes could be ascribed to the higher hydrophilicity. These membranes are hence expected to have superior fouling properties.

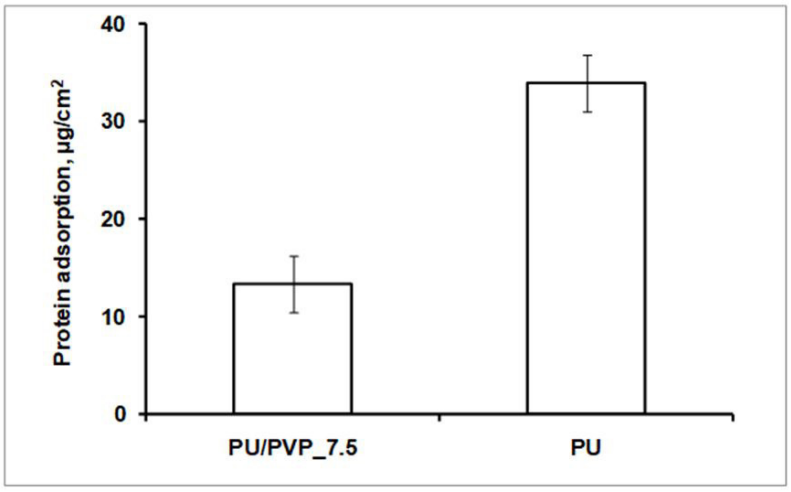

Figure 6: Protein adsorption on PU and PU/PVP honeycomb membranes

Surface chemistry and topographic features of a substrate have great influence on cell growth and proliferation [31]. In order to investigate the influence of honeycomb patterns on cell adhesion and growth behaviours, MSC cultured up 24h on honeycomb-patterned and flat PU/PVP_7.5 membranes and compared (Figure 7). While the cells on the flat films formed a monolayer, lesser number of cells was found on the honeycomb membranes (pore size 3μm). The influence of ordered honey-comb membranes on the cell morphology, proliferation and functions have been reported by many researchers [32,33]. Whilst the honeycomb structure aids the proliferation, viability and functions of certain cell types (e.g. endothelial cells, hepatocytes, cardiac myocytes, neural stem cells), the growth and motility of certain others (cancer cell) have been found to be inhibited by these ordered structures [34].

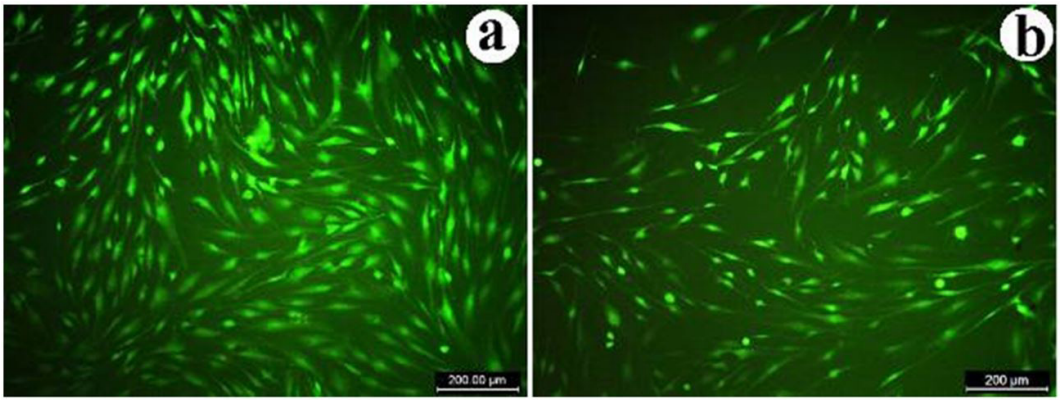

Figure 7: Fluorescent optical micrographs of MSC cells attached on (a) flat PU/PVP_7.5 and (b) honeycomb PU/PVP_7.5 membranes

Although, at this stage of investigation, we have no clear evidence to elucidate the reasons for which the cells adhering to the honeycomb films showed lowered growth, we suggest the following possibilities. Some MSC might have suffered mechanical stress after they got trapped into the pores of honeycomb films which inhibited their proliferation due to the lack of sufficient space [34]. It has also been reported that cell adhesion and progression could be inhibited if the cells fail to override the surface discontinuity produced as a result of patterning [30]. Nevertheless, we find this observation interesting and valuable as it qualifies these membranes to be used in applications such as immune-isolation and non-adherent membranes.

Conclusion

This study demonstrated that BF technique could be successfully used for the fabrication honeycomb membranes of PU. The choice of polymer and surfactant concentration, and the relative humidity were found to influence both the microstructure of the resultant membranes formed. Incorporation of PVP as a surfactant not only aided in formation of honeycomb structure in concentrated polymer solution but also improved the fouling properties of the resultant membranes. The next phase of investigations would thus focus on the transport and fouling properties of these membranes and also to uncover the mechanisms underlying cell growth inhibition by the honeycomb membranes.

Acknowledgements

Author SV acknowledges the help rendered by Dr. Dhanya Gangadharan (Biological studies) and Dr. Sajeev Sivaraman (AFM).

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