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Evaluation of physicochemical and biological properties of chitosan/poly (vinyl alcohol) polymer blend membranes and their correlation for Vero cell growth

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ABSTRACT

The blend membranes with varying weight ratios of chitosan/poly (vinyl alcohol) (CS/PVA) (1:0, 1:1, 1:2.5, 1.5:1, 1.5: 2.5) were prepared using solvent casting method and were evaluated for their potential application in single-use membrane bioreactors (MBRs). The physicochemical properties of the prepared membranes were investigated for chemical interactions (FTIR), surface morphology (SEM), water uptake, protein sorption (q_e), ammonia sorption and growth kinetics of Vero cells. CS/PVA blend membrane having weight ratio of 1.5:1 had shown enhanced membrane flexibility, reduced water uptake, less protein sorption and no ammonium sorption compared to CS membrane. This blend membrane also showed comparatively enhanced higher specific growth rate (0.82/day) of Vero cells. Improved physicochemical properties and growth kinetics obtrude CS/PVA (1.5:1) as a potential surface for adhesion and proliferation with possible application in single use membrane bioreactors. Additionally, new insight explaining correlation between water holding (%) of CS/PVA (1.5:1) blend membrane and doubling time (t_d) of Vero cells is proposed.

1. Introduction

The growing trend towards the use of disposable bioreactors, either driven by market need or regulatory constrains have provided an impetus to a paradigm shift toward wider acceptance of disposable bioreactor (Eibl, Kaiser, Lombriser, & Eibl, 2010). The cultivation container of such bioreactors, unlike traditional bioreactors of glass and steel, are made from FDA approved materials (Eibl & Eibl, 2006). Such bioreactors were widely used for production of therapeutics and commercially important biomolecules using eukaryotic and prokaryotic source organism.

The choice of bioreactor for cell culture is often decided based on cell-specific demands, engineering aspects, as well as economic and regulatory considerations. It is pertinent to use a bioreactor with least cell damage (due to aeration and agitation), well controlled environment, low level of toxic metabolite accumulation, higher yield of cell biomass and product, surface for adherent cells and consideration for scalability (Catapano, Czermak, Eibl, Eibl, & Pörtner, 2009). Such outcomes can be easily achieved in membranes bioreactors, which are effectively used to segregate cells, operated either in static conditions or in perfusion mode. Also, membranes are freely permeable to nutrients and products and provide a suitable surface for high densities anchorage-dependent cells culture. These features ultimately yield higher productivity concomitant with lower downstream processing costs (Chu et al., 2009). Membrane bioreactors have been successfully used for production of biomolecules and in various tissue engineering applications (Popović & Pörtner, 2012). Albeit such advantages, limited reports are available on design and fabrication of disposable membrane bioreactors (Shastri, 2003). This may be due to limitations in the type of available polymers with desired physical and chemical properties that can provide a suitable surface for cell adhesion and proliferation. Also existing surface material are costly and a concern during disposal. Therefore, strategic designing

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of disposable membrane bioreactors may demand the use of cost effective alternative biocompatible membrane material.

Different natural and synthetic polymers have been used for cultivation of anchorage dependent cells (Catapano et al., 2009). Synthetic polymers for adherent cell culture provide excellent results but get restricted in applications due to membrane fouling, high protein sorption and non-degradability (Boributh, Chanachai, & Jiraratananon, 2009). Recent reports have highlighted the use of chitosan (CS) coated membranes in membrane bioreactors for their antifouling (Le Roux, Krieg, Yeates, & Breytenbach, 2005; Wang, Yang, Meng, Zhang, Xue, & Fu, 2010), high flux (Wang, Yang, & Zhang, 2010) and antimicrobial properties (Hilal, Khayet, & Wright, 2012). CS, being one of the versatile biopolymer on earth has been extensively used for different biomedical and biotechnological applications. However, its application as a membrane material is limited due to physical and chemical properties of the polymer (Hamilton et al., 2007).

Current study evaluates the use of chitosan and PVA blends as an alternative surface material for Vero cells culture. The choice of CS based membrane may be justified based on its abundant availability, low cost, biocompatibility and biodegradability (Chuang, Young, Yao, & Chiu, 1999; Rhazi et al., 2002; Silva, Caridade, Mano, & Reis, 2013; Yang, Zhou, Chuo, Wang, & Yu, 2007; Yuan, Zhang, Yang, Wang, & Gu, 2004). In this study, Vero cell line has been used as a model system due to its wide acceptance as FDA approved cell line for biopharmaceutical manufacturing. A series of CS and PVA blend membranes (henceforth designated as CS/PVA) were prepared by varying weight percentage of the two polymers and were further characterized for physical and chemical properties. Comparative evaluations of these membranes as alternative surface for Vero cell growth and proliferation was also analyzed.

2. Materials and method

2.1. Materials

Chitosan (CS) (medium molecular mass; degree of deacetylation ~91%) was purchased from Meron Biopolymers, India. Polyvinyl alcohol (PVA) (Molecular weight 140 kDa), Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), Trypsin-EDTA and Bovine serum albumin (BSA) were procured from Himedia Laboratories, India. Acetic acid (AA) was purchased from CDH, India. All other reagents were of analytical grade and used without any further purification.

2.2. Membrane preparation

Stock solutions of CS (3% w/v) and PVA (5% w/v) were mixed in 2% v/v acetic acid to varying weight ratios of 1:0, 1:1, 1:2.5, 1.5:1 and 1.5: 2.5, stirred overnight at room temperature, and poured into 50 mm diameter polystyrene petridishes for membranes casting. The solvent was evaporated in incubator at 40 °C till dried. The dried membranes were recovered from the petriplates and then neutralized by rinsing with 1N NaOH, followed by rinsing in deionized water and finally re-dried at room temperature (Sharma et al., 2015).

3. Characterization

3.1. ATR-FTIR analysis

The attenuated total reflectance mode Fourier transform infrared (ATR-IR) spectra of the membranes were recorded using BX-II FTIR spectrophotometer (Perkin Elmer, USA) in the range of 4000–650 cm^{-1} with a resolution of 1 cm^{-1}. The polymer blending in different membranes was analyzed comparing FTIR spectra of chitosan, PVA and CS/PVA membranes.

3.2. Water holding

Water holding (swelling behavior) is an important parameter for analysis of water absorption of the membrane. Swelling behavior of the CS/PVA blend membrane were determined by immersion of 1×1 cm^2 excised pieces in 1 mM PBS (pH 7.4) at 37 °C for 30 min. The water holding (%) was calculated as shown in Eq. (1) (de Souza Costa-Júnior, Pereira, & Mansur, 2009).

$$\text{Water holding}(\%) = \frac{W_s - W_d}{W_d} \quad (1)$$

where, W_s = weight of membrane after swelling; W_d = weight of dry membrane.

3.3. Adsorption kinetics

Adsorption kinetics of protein (BSA) and ammonia (ammonium chloride) were studied using 6.5 mm (diameter) discs incised from all the membranes. The incised discs were kept in 96 well plate with varying concentrations of NH_4Cl (2–10 mM). The time dependent studies on change in ammonia concentration in solution were analyzed using modified Weatherburn method (Weatherburn, 1967). Briefly, 10 μl of the sample was withdrawn from each well every 5 min, (for 40 min) mixed with 60 μl salicylate solution and 60 μl bleach solution and the absorption was measured at 650 nm. The protein (BSA) sorption on different membranes was studied using the method of Deniz et al. (Tanyolaç, Sönmezışık, & Özdural, 2005), with slight modifications. Briefly, varying concentrations of 300 μl BSA solution (0.2, 0.4, 0.6, 0.8 and 1 mg/ml) were incubated with 6.5 mm incised membrane discs, pre-soaked in 1 mM PBS buffer (pH 7.4), and batch adsorption studies were performed for 40 min at 37 ± 2 °C in a 96 well plate. The samples were recovered in every 5 min and analyzed for protein concentration using Bradford method.

3.4. Protein (BSA) adsorption studies

The time dependent variation in the concentrations of BSA in solution were determined using Bradford method (Bradford, 1976). BSA concentration was calculated using standard graph of BSA concentration versus absorbance (595 nm). Sorption of BSA on membranes was calculated as time dependent decrease in BSA concentration in solution. The experiments were performed in triplicates and the average values were recorded. Data was interpreted using pseudo first order and second order adsorption kinetics as reported previously (Ho & McKay, 2000; Langergren, 1898). The equation of Langergren (1898) was used as the first order kinetic model as given in Eq. (2).

$$\log(q_e - q_t) = \log q_e - \left(\frac{k_1}{2.303}\right) t \quad (2)$$

where, q_e is the amount of adsorbed protein at equilibrium (mg/mm^2), q_t is the amount of adsorbed protein on the adsorbent (mg/mm^2) at time t and k_1 is the adsorption rate constant for first order kinetics. The integral form of the pseudo-second order kinetics (Ho & McKay, 2000) is expressed in Eq. (3):

$$\frac{t}{q_t} = \frac{1}{k_2 q_e{}^2} + \frac{1}{q_e t} \quad (3)$$

where, k_2 is rate constant of Pseudo-second order kinetics (g/mm^2).

The plot of t/q_t against t gives a linear relationship from which q_e and k_2 can be determined from the slope and intercept of the plot, respectively.

3.5. Cell culture and seeding

Vero cells were grown in DMEM containing 10% (v/v) FBS in a T-25 culture flask and maintained in incubator, equilibrated with 5% CO_2 at 37 °C. Further, the wells of a 24 well polystyrene plate were blocked with BSA solution (3% w/v) for overnight followed by rinsing with autoclaved distilled water. Uniform sized membrane pieces ($1 \times 1\ cm^2$; 70 μm thickness), sterilized overnight with 70% alcohol and rinsed extensively with sterile 1 mM PBS, were placed in the pre-blocked wells and equilibrated overnight in DMEM medium. Cell growth and proliferation studies were performed for 5 days with initial cell density of 10^4 cells/cm^2 in a humidified atmosphere with 5% CO_2 at 37 °C. Results were analyzed using MTT assay.

3.6. Cell activities

The adhesion and proliferation of the Vero cells on membranes were determined using MTT assay after 5 days of cell culture. A modified MTT assay was used to study proliferation of Vero cells on membranes (Doyle & Bryan, 1998). Briefly, equilibrated membranes were washed with 1 mM PBS (pH 7.4) and suspended in 500 μl DMEM media followed by supplementation with 50 μl MTT (5 mg/ml) solutions. After 3 h of incubation at 37 °C, 500 μl DMSO (dimethyl sulfoxide) was added to stop the reaction and membranes were sonicated for 10 min to dissolve formazan crystals. The optical density of the formazan was measured at 570 nm using ELISA plate reader (Bio-Rad, USA). All experiments were repeated three times and results were expressed as mean ± standard deviation. Specific growth rate of cells (μ), on polymer surface were determined as reported previously (Eibl & Eibl, 2009) using Eq. (4).

$$N_t = N_0 e^{\mu(t_2 - t_1)} \tag{4}$$

where, N_t and N_0 are viable Vero cells/cm^2 during exponential phase of cell growth at time t_2 and t_1 respectively. Specific growth rate (μ) represents an exponential change in viable Vero cell count under the defined culture condition. It is also indicative of the doubling time (t_d) of an organism by Eq. (5).

$$t_d = \frac{0.639}{\mu} \tag{5}$$

3.7. Scanning electron microscopy

The surface topography of dried CS/PVA (1.5:1) blend membranes and Vero cell adhesion on this membrane was analyzed after 3 days culture using SEM micrograph (EVO 50 and EVO 18 special edition, Zeiss). Samples were pre-fixed with 1% glutaraldehyde for 1 h to study Vero cell adhesion. All the membranes were sputter coated with gold and examined using scanning electron microscope.

3.8. Mechanical properties

The selected blend membrane (based on physicochemical properties) was analyzed for its mechanical properties using a tensile

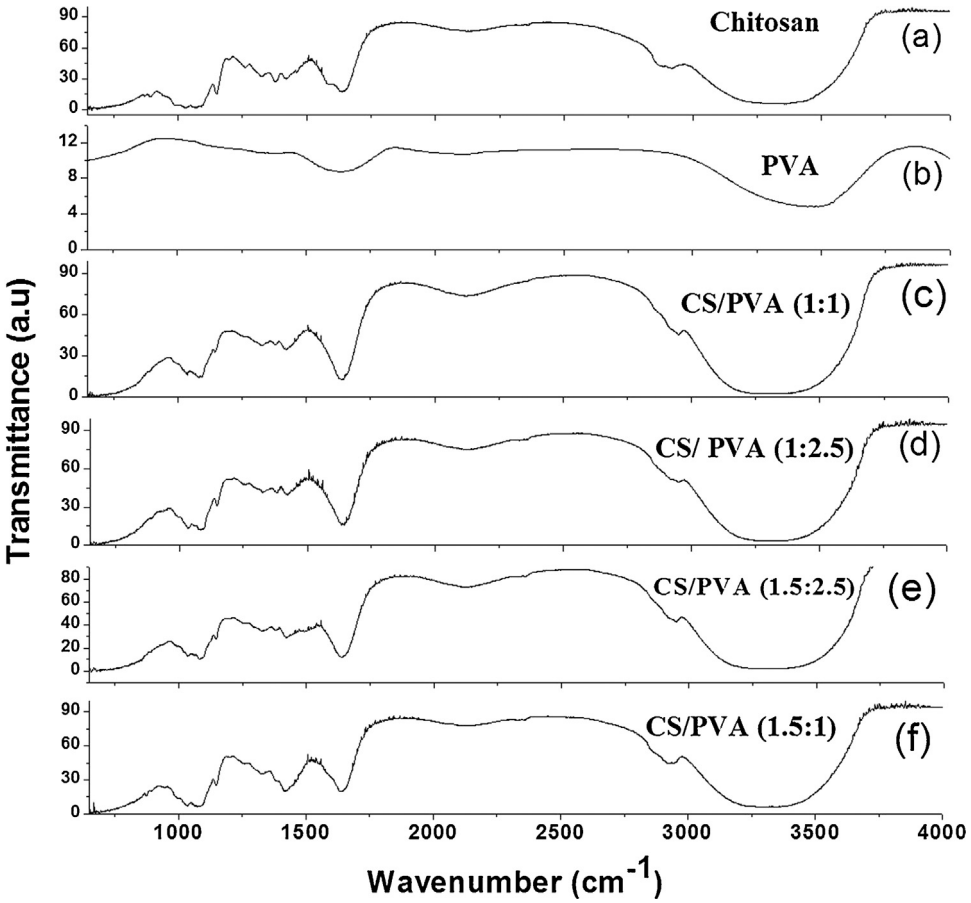

Fig. 1. FTIR spectra of the (a) CS membrane, (b) PVA membrane, (c) CS/PVA (1:1) blend membrane, (d) CS/PVA (1:2.5) blend membrane, (e) CS/PVA (1.5:2.5) blend membrane, (f) CS/PVA (1.5:1) blend membrane.

testing device (Instron Microtensile Tester, Model 5848, Singapore). The elasticity was determined as the distance the probe traveled before breaking the membrane. For analysis, membranes strips (6 mm × 25 mm) were clamped and stretched at a speed of 50 mm/min. The tensile strength (TS) was expressed in MPa. All measurements were done in triplicates and results were expressed as mean ± standard error.

4. Results and discussions

4.1. Characterization of membrane

4.1.1. ATR-FTIR analysis

The ATR-FTIR spectra of CS, PVA and CS/PVA membranes were compared for their signature peaks and chemical interactions (Fig. 1a–f). The FTIR spectra of CS/PVA membranes exhibited absorption bands of both chitosan and PVA within the blend membranes. FTIR spectrum of pure CS membrane (Fig. 1a) showed the characteristic vibration peaks in the typical fingerprint region around 893 and 1165 cm^{-1} corresponding to saccharide structure, the repeating unit of chitosan (Costa-Júnior, Barbosa-Stancioli, Mansur, Vasconcelos, & Mansur, 2009; Mansur & Costa, 2008; Sharma et al., 2015). The spectra of all CS/PVA blend membranes (Fig. 1c–f) showed —O—H and —N—H stretching about 3470 cm^{-1}, —C—H stretching about 2947 cm^{-1} and —C—O stretching around 1079 cm^{-1}. The shifting and broadening of ~3225 cm^{-1} mode for individual constituents i.e., CS and PVA (for blend membranes) to 3217 cm^{-1} indicate a possible overlapped stretching between the $—NH_2$ (in chitosan) and the —OH (in PVA) groups and hence, reflecting physical blend and chemical interactions which is evident in all blend membrane spectra (Anicuta, Dobre, Stroescu, & Jipa, 2010; Vimala et al., 2011).

4.1.2. Water holding (%)

The water holding of membranes reflect capability of the membrane to hold aqueous medium which assist cell growth albeit its limitation in retention of membrane shape and membrane fouling (Luangbudnark, Viyoch, Laupattarakasem, Surakunprapha, & Laupattarakasem, 2012). Table 2 shows the swelling behavior of different membranes. Results showed least water hold up by CS/PVA (1.5:1). Reduction in water holding capacity is a significant parameter to retain their shape, size and reduce the chances of membrane fouling during bioprocess applications. It was also observed that the supplementation of CS with PVA decrease water holding capacity compared to pure CS membrane (Sharma et al., 2015). CS/PVA (1.5:1) blend membrane showed least water holding and could retain its shape after being immersed in culture media, supporting the possible application of the membrane in MBRs. Such disparity

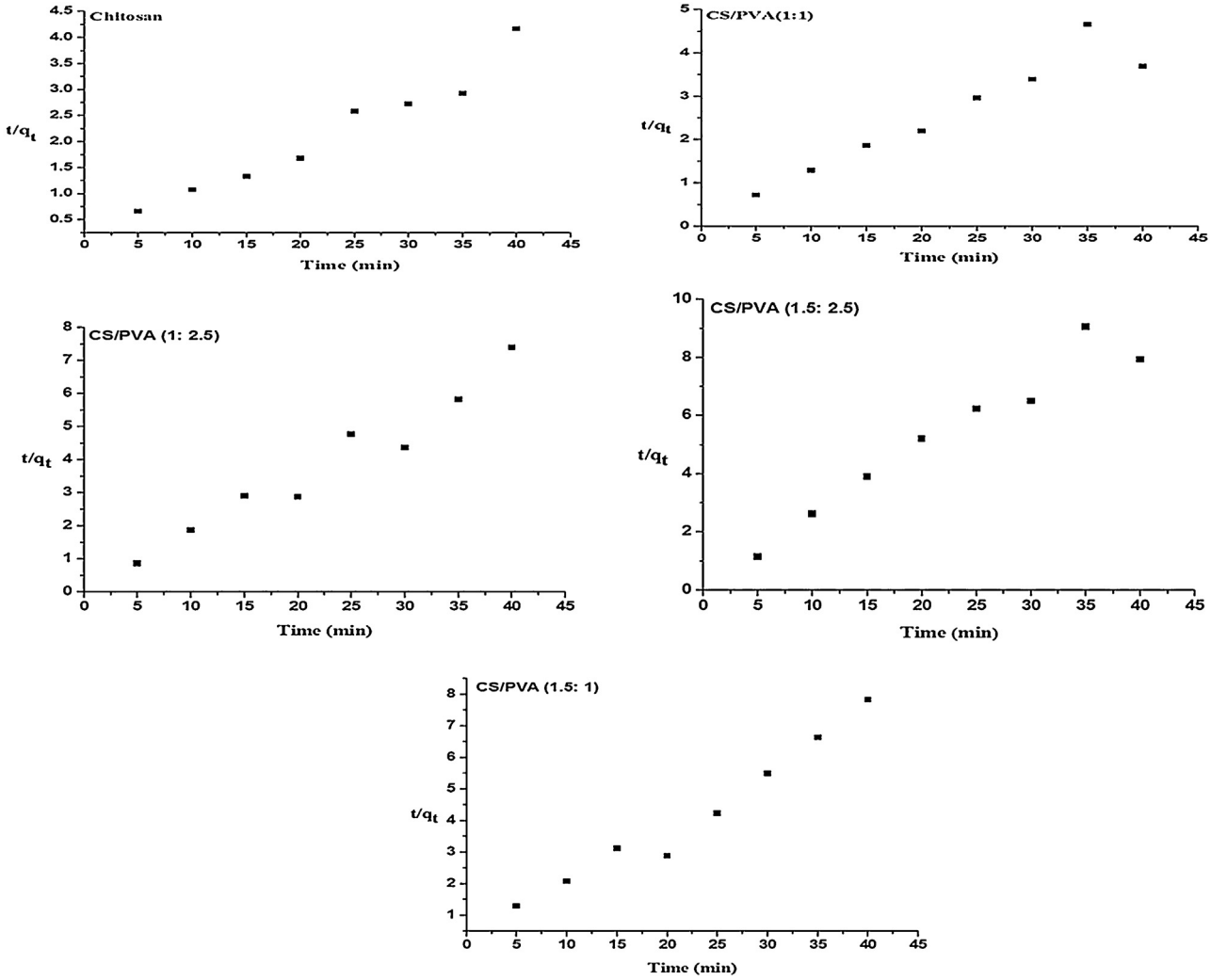

Fig. 2. Adsorption kinetics of BSA on (a) pure CS, (b) CS/PVA (1:1) blend membrane, (c) CS/PVA (1:2.5) blend membrane, (d) CS/PVA (1.5:2.5) blend membrane, (e) CS/PVA (1.5:1) blend membrane.

Table 1
Comparison of the pseudo-second order sorption kinetics coefficient correlation (R^2), adsorption rate constant (k_2) and experimental (q_e) values.

Samples	Equilibrium protein sorption (q_e) (mg/mm^2)	Rate constant of pseudo-second order kinetics (k_2) (g/mm^2)	R^2 (Coefficient correlation)
CS	10.81	0.137	0.95
CS/PVA(1:1)	9.80	0.035	0.91
CS/PVA(1:2.5)	6.45	0.096	0.94
CS/PVA(1.5:1)	5.46	0.472	0.96
CS/PVA(1.5:2.5)	4.74	0.076	0.94

may be attributed to variation in the inter- and intra- molecular interactions within the blend membranes (Alhosseini et al., 2012).

4.2. Adsorption kinetics study

All membranes were analyzed for ammonia and BSA adsorption kinetics. Ammonia is one of the toxic by-products of L-glutamine metabolism with deleterious effect on the cell growth. Thus, it is pertinent for membrane surface to have low ammonia sorption (Butler & Christie, 1994). In this study, ammonia sorption studies performed on pure CS and CS/PVA blend membranes did not show any significant ammonia sorption by membranes.

On the contrary, previous studies have reported that proteins sorption within membrane pores and on surface lead to membrane fouling (Labbe, Quemerais, Michel, & Daufin, 1990; Mutamim, Noor, Hassan, Yuniarto, & Olsson, 2013; Zhao, Wu, Wang, Zhao, & Li, 2000). Membrane fouling curtails severely the economical and practical implementation of membrane processes (Changsheng, Xingyan, Dewei, & Jian, 1996). Such membranes are also prone to microbial attachment and degradation, affecting flux of liquid through the membranes (Wang, Yang, Meng et al., 2010). In this study, protein sorption results were well fitted using pseudo second order rate Eq. (3) (Fig. 2a–e). The results of protein sorption kinetics were used to describe the phenomena of interparticle equilibrium protein sorption (q_e) and the kinetic rate constant on chitosan and CS/PVA blend membranes. It has been observed that variation in weight ratios of parent polymers significantly affected the adsorption capacity of BSA by the membranes (Table 1). CS/PVA blend membranes with weight ratios of 1:1 and 1:2.5 showed around 10% and 40% reduction respectively in protein sorption compared to CS membrane. Further, CS/PVA blend membranes with weight ratios of 1.5:1 and 1.5:2.5 resulted in reduction of equilibrium protein sorption by around 50% and 56% respectively. Reduction in equilibrium protein sorption capacity may be associated with change in bulk properties of the membrane, possibly due to interaction within the two polymers as analyzed using FTIR spectroscopy.

4.3. Growth of Vero cells on polymer membranes

Cell adhesion and proliferation on membrane surface is known to be influenced by surface properties such as topography and roughness (Lord, Foss, & Besenbacher, 2010). The effect of polymer blending on cell count (growth rate) of Vero cells was evaluated, as displayed in Fig. 3. Results in Table 2 show higher specific growth rate (0.82/day) of Vero cells on CS/PVA (1.5:1) in comparison to other CS and CS/PVA membranes (Sharma et al., 2015).

4.4. A mathematical correlation

A mathematical correlation between water holding (%) of membrane and doubling time (t_d) was proposed based on experimental results using MATLAB (ver.7.10.0.499). Results showed linear polynomial correlation between water holding and doubling time of Vero cells. Fig. 4 shows curve fitting data and its correlation with

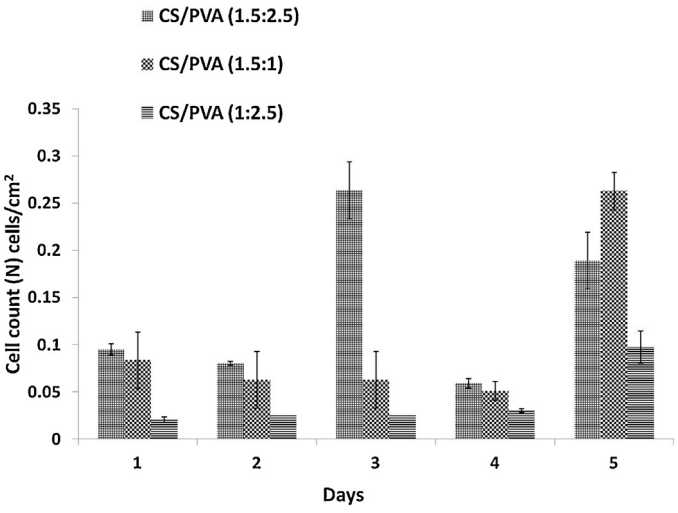

Fig. 3. Vero cell count (cells/cm^2) on CS/PVA (1.5:2.5), CS/PVA (1:2.5) and CS/PVA (1.5:1) blend membranes.

calculated data. The linear polynomial correlation between water holding and doubling time is shown by mathematical Eq. (6).

$$\text{Doubling time}\,(t_d) = 0.039(\text{Water holding}) - 0.432 \quad (6)$$

The graphical correlation shows experimental observations to be closed to 20% of the predicted values. Experimental observations are well fitted with the proposed model in Eq. (6) and show comparatively insignificant deviation from the theoretical values (Table 2). Based on Eq. (6), it can be proposed that membrane with very low water holding capacity (~11%) will not allow doubling of Vero cells on CS/PVA (1.5:1) blend membrane. The outcome suggests that though high water holding is not a desired feature, reduction of water holding to a very low level may also affect cell proliferation.

Unlike previous reports where decrease water holding capacity by chitosan based scaffolds have been directly correlated with

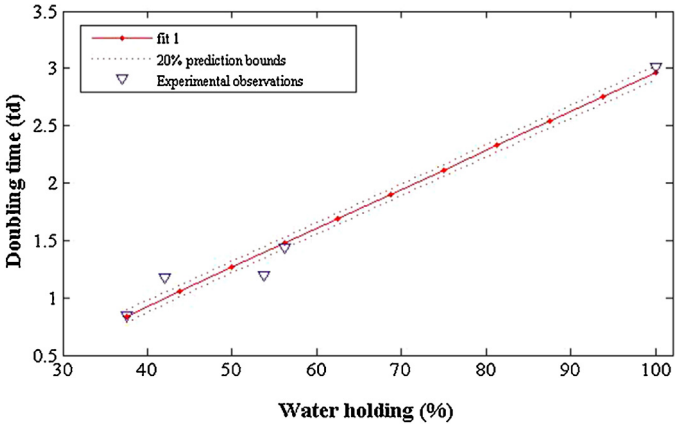

Fig. 4. Curve fitting data showing a linear polynomial correlation between water holding (%) and doubling time (t_d).

Table 2
Correlation between water holding and doubling time based on mathematical model.

Samples	Water holding (%)	Specific growth rate (μ) (day^{-1})	Doubling time (t_d) (day)	
			Theoretical (based on model)	Experimental
CS	100[a]	0.23[a]	3.34	3.01
CS/PVA (1:1)	53.8[a]	0.58[a]	1.39	1.2
CS/PVA (1:2.5)	56.2	0.48	1.47	1.44
CS/PVA (1.5:1)	37.5	0.82	0.83	0.85
CS/PVA (1.5:2.5)	42	0.59	0.99	1.17

[a] Results from previous studies (Sharma et al., 2015).

proliferation of fibroblast cells (Lawrence & Madihally, 2008; Marois & Guidoin, 1999). Findings in current study revealed decrease in doubling time with decrease water holding capacity. Reduced water holding capacity is often a desired property for the use of membrane in chemical processes. Previous studies have also shown water holding phenomenon as representation of membrane porosity where higher water holding has been attributed to large pore size. This property is of significant importance in biomedical applications and scaffold fabrications. However, such properties create heterogeneity in bioprocess operations by affecting the overall working volume. Hence, it is pertinent to design a membrane with comparatively low water holding capacity. Porosity is also correlated with enhanced in-growth of primary cells on scaffolds (Lawrence & Madihally, 2008), a parameter less significant in process operations due to mass transfer limitation of nutrient and other abiotic factors to the cells growing inside the membrane. Results showed a linear increase in doubling time of Vero cells with increase in water holding (%) by CS/PVA membranes ($R^2 = 0.96$). Higher porosity may be associated with reduced cell matrix interaction on the surface of the membrane thereby decreasing the rate of proliferation of anchorage dependent cells. This is a first report that gives a new insight to correlation between water holding of CS/PVA membrane and doubling time of Vero cells. Variation in water holding capacity is primarily due to varying ratios of two parent polymers. However, the roles of other abiotic and biotic parameters are not ruled out.

4.5. Mechanical characteristics

MBRs are often operated with high fluid flux that demand the use of membranes with higher mechanical stability (Visvanathan & Aim, 2002). Chitosan often find limitations in applicability due to its poor mechanical properties (Oh & Hwang, 2013; Parida, Nayak, Binhani, & Nayak, 2011). The comparison of pure CS and CS/PVA (1.5:1) membranes suggested that addition of PVA into CS altered the mechanical properties of the blend with comparatively higher flexibility. This may be attributed to increased mobility of the polymers interactions which might produce strong hydrogen bonding and consequently changes mechanical properties (Zhuang, Li, Fan, Lin, & Hu, 2012). Mechanical strength of

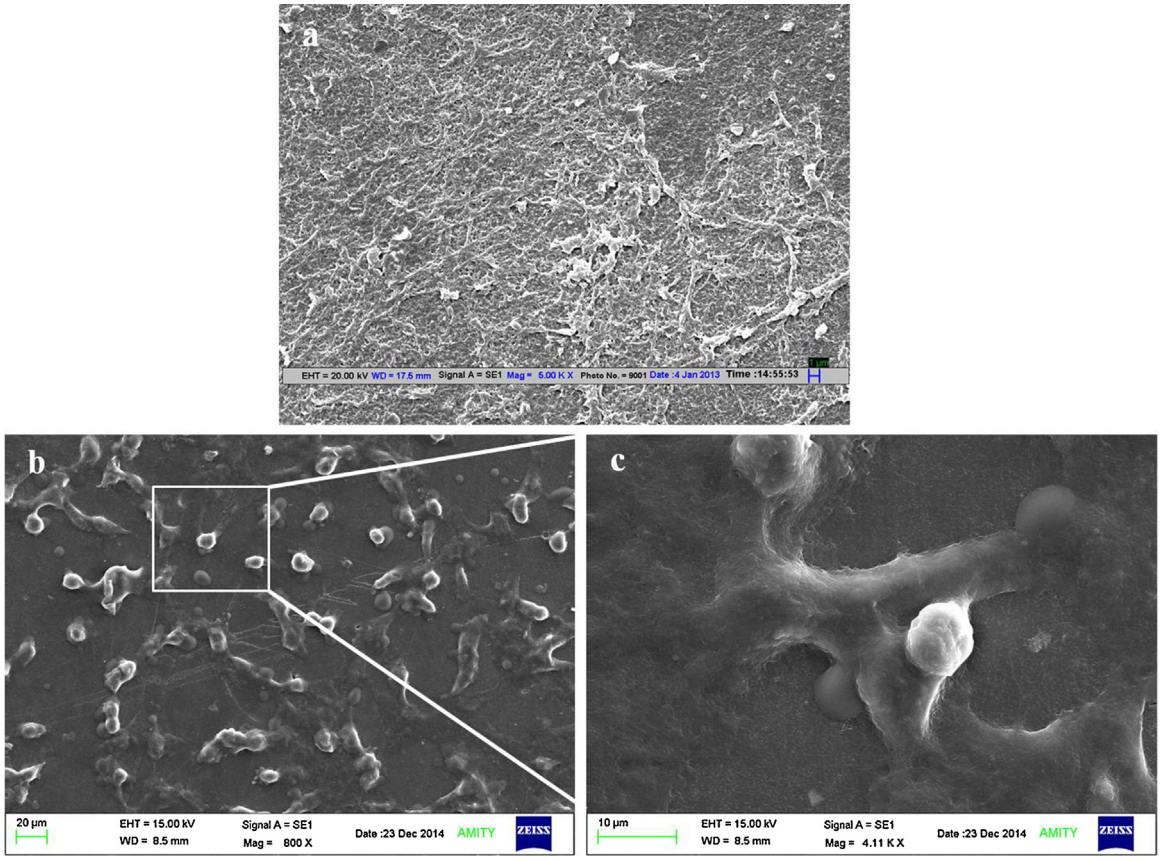

Fig. 5. Scanning electron micrographs showing surface morphologies of (a) CS/PVA (1.5:1) blend membrane at 5 KX (scale bar, 1 μm), (b) CS/PVA (1.5:1) blend membrane with Vero cells at 0.8 KX (scale bar, 20 μm) (c) filopodia connection of Vero cells on CS/PVA (1.5:1) at 4.1 KX (scale bar, 10 μm).

CS/PVA (1.5:1) membrane showed an increase in elasticity from 1.23 ± 0.08 to 17.6 ± 0.04 mm and reduction in tensile strength from 32.47 ± 1.79 MPa to 16.29 ± 1.86 MPa as compare to CS membrane (Sharma et al., 2015).

Overall results of mechanical tests suggested that blending of CS and PVA improved mechanical properties of the membrane. It can also be suggested that along with the choice of polymers, their weight ratio also plays a pivotal role in imparting changes in the physical and chemical properties of the blend membranes based on their chemical interactions. This may be attributed to interactions between —OH and $—NH_2$ groups in these two polymers (Parida et al., 2011). The mechanical properties of CS/PVA (1.5:1) blend membrane were comparable to other CS/PVA blends, reported previously (Bahrami, Kordestani, Mirzadeh, & Mansoori, 2003).

4.6. Surface morphology of the membrane

The selected membranes were further examined by scanning electron microscopy (SEM) to analyze the surface morphology of the membrane. The surface of CS/PVA (1.5:1) before cell culture appeared porous and filamentous (Fig. 5a). SEM micrograph (Fig. 5b and c) revealed normal morphology and filopodial connections of the Vero cells, cultured on CS/PVA (1.5:1) blend membrane. Previous studies have shown that along with surface charge and chemical properties of surface, physical properties (surface rugosity) also plays a significant role in cell adhesion and proliferation (Alves, Ginani, Silva, Alves, & Barboza, 2011; Da Silva et al., 2010; Sá et al., 2009).

5. Conclusions

The properties of the casted CS/PVA blend membranes were mainly influenced by the weight percentage of CS and PVA. The blending of CS and PVA improves membrane elasticity, reduce maximum protein sorption capacity and reduced water sorption. Low water holding and protein sorption capacity of CS/PVA (1.5:1) blend membrane with concomitant improvement in elasticity obtrudes it to be a suitable membrane surface in MBRs for better specific growth of Vero cells. CS/PVA may thus provide a cost effective disposable solution to synthetic membranes in MBRs. Apart from their use for Vero cell proliferation, the use of CS/PVA blend membranes may also be extended to evaluate their potential application in commercial, bio manufacturing and remediation sectors. However, the variations in physical, chemical and biological properties of the blend membranes due to variation in molecular mass of parent polymers and polymers weight percentage cannot be ruled out and may be significant importance in their application.

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