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Desalination 234 (2008) 134–143
DESALINATION
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Immobilization of l-lysine on dense and porous poly(vinylidene fluoride) surfaces for neuron culture

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Received 10 July 2007; accepted revised 28 September 2007

Abstract

Microporous poly(vinylidene fluoride) (PVDF) membranes with either dense or porous surface were prepared by isothermal immersion-precipitation of a casting solution in coagulation baths of different strengths. Onto the membrane surface, an amino acid (l-lysine) was immobilized by a dual-step chemical process. First, the membrane was grafted with poly(acrylic acid) (PAA) by means of plasma-induced free radical polymerization. Then, l-lysine was covalently bonded to the as-grafted PAA chains with the aid of a water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). The highest attainable graft yield of PAA on PVDF membrane reached up to 0.64 mg/cm^2. For immobilization of l-lysine on the membrane, the yields were found to depend on factors, such as concentration of EDC, activation time, and pH value. The maximal attainable immobilization yield was 0.65 μg/cm^2. Furthermore, pheochromocytoma (PC12) cells were cultured on l-lysine/PAA/PVDF membranes. It was found that both the amount of l-lysine on the membrane and the surface structure had a marked influence on the cell activity. Thus, the present results could be useful for the development of strategies to promote the re-growth and regeneration of tissue in the nervous system.

Keywords: Poly(vinylidene fluoride); Porous; Membrane; Immobilization; l-lysine

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Presented at the Fourth Conference of Aseanian Membrane Society (AMS 4), 16–18 August 2007, Taipei, Taiwan.

doi:10.1016/j.desal.2007.09.079

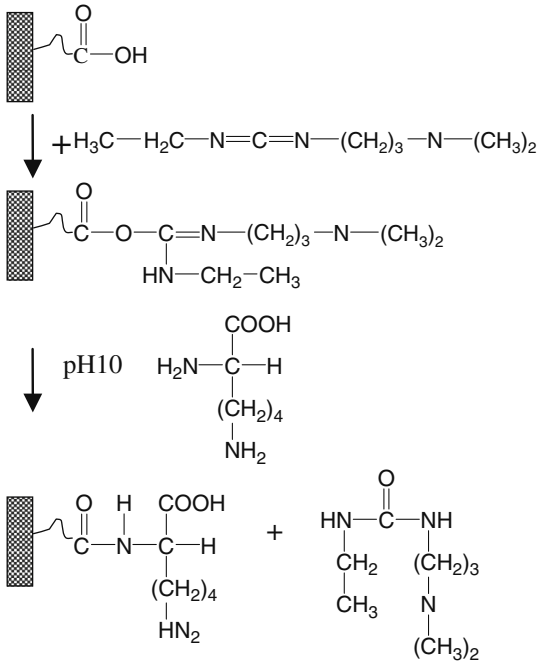
1. Introduction

Poly(vinylidene fluoride) (PVDF) is an acid resistant, chemically inert, and mechanically strong high performance plastic, which is used extensively in a wide diversity of industries, such as electronics, food, pharmaceuticals, etc. Microporous PVDF membranes have been commercialized for many years by Millipore Corp. as an important fine separation medium. These membranes are prepared by the so-called immersion-precipitation technique [1,2]. It is of great interest to investigate how significant the surface morphology affects the efficiency of chemical immobilization of biomaterial on these membranes.

Plasma induced free radical polymerization is a useful technique for grafting acrylic polymers onto the surface of an inert polymeric substrate such as, Teflon, PVDF, PE, etc. [3–5]. Polylysine is a biopolymer that is often applied onto a substrate to enhance the cellular compatibility for neuronal cultures [6]. However, the binding strength for this type of coating, as is based on physical interaction, is relatively weak so that polylysine can detach during the course of applications. To resolve this problem, l-lysine is immobilized by covalently bonding to the membrane surfaces. It is hoped that the immobilized l-lysine will mimic the behavior of the very expensive polylysine, and act as an effective medium for neuronal culture.

Immobilization of l-lysine on PVDF membranes was made possible by adopting poly(acrylic acid) (PAA) as an inter-linkage between PVDF and l-lysine. First, PAA was grafted on PVDF membrane by means of plasma-induced polymerization [4,5]. Subsequently, l-lysine was bonded to the carboxylic acid groups of the grafted PAA with the aid of a carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). As shown in Scheme 1 [5].

Immobilization of l-lysine on poly(ethylene-co-vinyl alcohol) dense films has been investigated by Young et al. [7]. However, because these films were nonporous, the immobilization yields were limited to a relatively low level. To increase the amount of immobilization and to see how significantly the surface morphology affects the immobilization yield, PVDF membranes with both dense and porous surface structures are employed in the present work. Also, factors that are crucial to grafting and immobilization reactions, such as reaction pH, monomer concentration, EDC-activation time, etc., are investigated to find the optimal condition for the synthesis of l-lysine/PAA/PVDF composite membranes. Finally, culture of pheochromocytoma (PC12) cells was performed. PC12 cells resemble adrenal chromaffin cells, which share many physiological properties of neurons [8,9]. The cellular activity on various membranes was compared, and it was found that



Scheme 1. Immobilization of l-lysine on a PAA-grafted PVDF membrane.

the amount of l-lysine on the membrane was closely related to the cell activity.

2. Experimental

2.1. Material

Poly(vinylidene fluoride) (Kynar 740, intrinsic viscosity = 0.881 dL/g, Mw = 254,000 g/mol) was supplied by Elf Atochem Inc. in pellet form. Dimethyl sulfoxide (DMSO, Acros, reagent grade, d = 1.096 g/mL) and water (distilled and de-ionized) were used as the solvent and nonsolvent, respectively, for membrane formation. Acrylic acid (AA, Acros, 99.5%, d = 1.051 g/mL) was vacuum distillated to remove trace of inhibitor MEHQ before grafting it onto PVDF membranes. L-lysine (98%) was purchased from Aldrich. 1-ethyl-3-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC, 98%) was purchased from Acros. All materials, except for acrylic acid, were used as received.

2.2. PVDF membrane formation

First, a casting dope was prepared by dissolution of PVDF in DMSO at 70°C to form a homogeneous solution containing 18% polymer. Following dissolution, the solution was cooled to room temperature (ca. 25°C), stood for 1 h, cast on a glass plate, and then immersed immediately into a nonsolvent bath to induce polymer precipitation. The formed nascent membrane was washed in a series of nonsolvents to extract residual DMSO. Subsequently the membrane was press-dried between sheets of filter papers at ca. 50°C. The morphology of the formed membrane was observed using a Field Emission Scanning Electron Microscope (LEO 1530, Carl Zeiss). Membrane samples dried in vacuum at 50°C were plated with a thin layer of Pt-Pd alloy and imaged at 1–3 KV with an in-lens detector.

2.3. PAA/PVDF composite membrane formation

PAA was covalently grafted onto the surface of a PVDF membrane by means of plasma-induced free radical polymerization. The membrane was plasma-irradiated (Argon at 0.4 mmHg) for 1 min and then exposed to the air for 10 min to form peroxide on the membrane surface, after which it was immersed directly in a deaerated AA/water solution (50 wt%) containing an appropriate amount of Mohr's salt. The grafting reaction was allowed to proceed for 5 h at 80°C [5]. To remove residual AA and un-grafted PAA, the formed PAA/PVDF composite membrane was shaken in a water bath for 24 h, during which water was replenished periodically. Thereafter, the membrane was rinsed with a large amount of water. Presence of PAA on PVDF membrane was confirmed by FTIR/ATR at the absorption band of the carbonyl group (1710 cm^{-1}). The amount of grafted PAA on the membrane was determined by titration. The PAA/PVDF composite membrane was put in a 0.01N NaOH solution, which was shaken for 24 h and then the solution was titrated against 0.001N HCl aqueous solution.

2.4. Immobilization of l-lysine

PAA/PVDF membrane (3 × 3 cm^2) was put in a buffer solution containing a known quantity of EDC (0.01–0.05 M) to activate the carboxylic acid group of PAA, as shown in Scheme 1. This reaction was performed at 4°C for a period of 0.5–6 h. The PAA-activated membrane was then put in a l-lysine solution (20 mg/mL) maintained at pH 10 and 4°C for 24 h to immobilize l-lysine on the membrane. The formed l-lysine/PAA/PVDF composite membrane was washed in a buffer solution (pH 10) for 24 h to remove residual un-reacted l-lysine and byproduct, isourea. The composite membrane was then rinsed in water and dried in an oven at 60°C. The immobilization yield of l-lysine was determined

by UV colorimetry using acid orange 7 (485 nm) as an indicator, as described in the literature [10].

2.5. Cell culture

The membranes were cut into a circular shape (1.5 cm in diameter) and secured in 24-well tissue culture polystyrene plates (TCPS, Corning, New York, USA) by placing a silicon rubber O-ring on top of each membrane. These membranes were sterilized with 70% alcohol under ultraviolet light overnight and then rinsed extensively with phosphate buffer solution (PBS). For comparison, an empty TCPS well with a silicon rubber O-ring was used as a reference, which was treated in the same way as the membrane-containing wells.

PC12 cells were cultured in an RPMI-1640 medium supplemented with 10% horse serum, 5% fetal bovine serum, penicillin G (100 IU/mL) and streptomycin (100 μg/mL), and maintained in a humidified atmosphere containing 5% CO_2 at 37°C, as recommended by Greene et al. [19]. Cells were routinely split at a ratio of 1:2 every 3–4 days by detachment from the culture flask with mild agitation and a stream of fresh culture medium. After a large number of cell divisions, cells were added to the culture wells at a density of 8×10^4 cells/cm^2.

Cell viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazol-ium bromide, Sigma) colorimetric assay at 4 h and 1 and 2 d after seeding cells on the membranes. For the MTT colorimetric assay, the cultures were incubated for 3 h at 37°C with 0.1 mL of MTT solution (2 mg/mL in PBS). The level of MTT reduction into blue/purple formazan crystals by mitochodrial dehydrogenase of viable cells was used to represent the level of cell metabolism. After MTT reduction, the medium was aspirated and the formed formazan was dissolved in 0.2 mL of DMSO and the plates were shaken for 20 min. The optical density of the formazan solution was read on an ELISA plate reader (EL×800, BIO-TEK) at 570 nm. The absorbance was proportional to the number of living cells present. Cell viability was determined from 4 to 6 independent cultures and expressed as mean ± standard deviation.

3. Results and discussion

3.1. Grafting of PAA on PVDF membranes

Morphologies of the membrane surfaces for grafting PAA are shown in Fig. 1. Fig. 1(a) depicts the top surface of the membrane ‘MS’, which was prepared by immersion- precipitation of a 18% casting dope in pure water. This surface appears dense and nonporous, and thus may be called a ‘skin’ of the membrane. Fig. 1(b) shows the bottom surface of the membrane ‘MP70’. It exhibits a porous morphology consisting of interconnected globular particles of a similar size (ca. 1–1.5 μm). These particles have

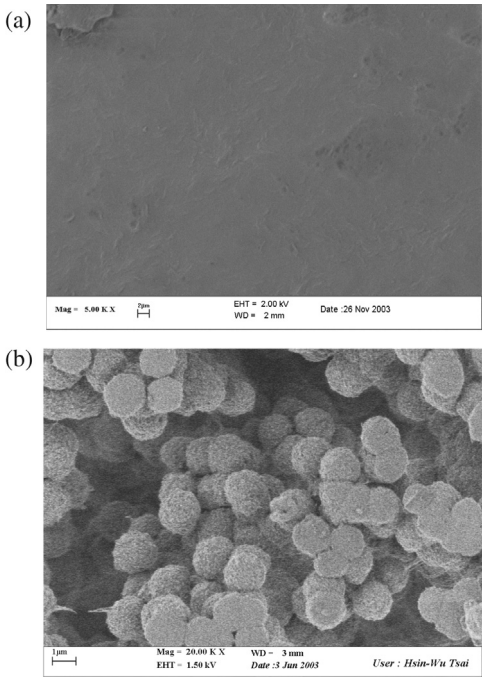


Fig. 1. Dense and porous PVDF membrane surfaces for grafting PAA. (a) MS; (b) MP70.

previously been identified as full spherulites of PVDF by means of FESEM, XRD, and POM analyses [11]. Between the particles are pores that form continuous channels in the framework of polymer host. It is worthwhile to note that the surfaces of the globules are rough and contain nano-sized pores [5]. As the porosity of 'MP70' is much higher than 'MW', one expects that the former would have a higher grafting yield. In some cases, membranes prepared by precipitation of the same casting dope in a 40% DMSO bath (i.e., membrane 'MP40') as the substrate for grafting PAA. The porous structure of this membrane's bottom surface is similar to that of 'MP70'. However, this membrane is mechanically stronger and thus is more suitable for subsequent l-lysine immobilization and cell culture.

PAA was grafted on the aforementioned membranes by the plasma-induced polymerization method. FTIR-ATR was employed to confirm the presence of PAA on the grafted membrane. As an example, Fig. 2 shows the spectra of pristine 'MP70' and 'MP70' grafted with different amounts of PAA. The absorption band at 1710 cm^{-1}, being assigned to the C=O stretching vibration, is evident for the PAA/PVDF composite membranes, and yet it is absent from pristine 'MP70'. Also, the intensity is higher for the membrane with a larger amount of grafted PAA. The grafting yields for a typical plasma-induced free radical polymerization depend upon a number of factors, such as plasma treatment time, plasma power, monomer concentration, reaction temperature, reaction time, etc. [3,12,13]. In the present work, attention has been focused on the following factors: concentration of Mohr's salt, morphology of the membrane surface, plasma power, and plasma treatment time.

In Fig. 3, grafting yields of PAA are demonstrated for reactions carried out using different concentrations of Mohr's salt. It can be seen that the grafting yields increase with increasing grafting time, irrespective of the concentration of the Mohr's salt. Given the fact that only a fixed amount of active sites were initially generated on the membrane surface by plasma bombardment, the gradual increase of PAA with time was thought to arise from growth of PAA chains rather than creation of new polymer chains. Because AA in the bulk solution could undergo free radical polymerization as well, the reaction

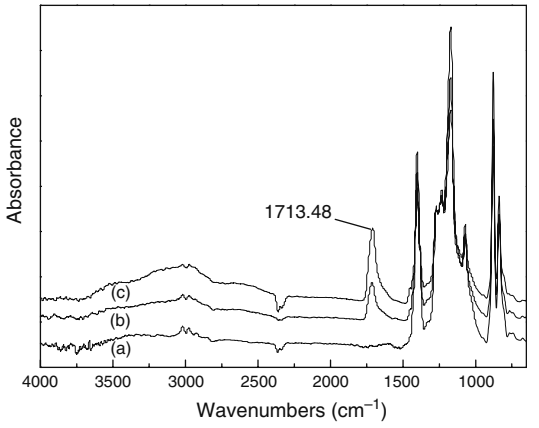


Fig. 2. FTIR-ATR spectra of primitive PVDF membrane and PAA/PVDF composite membranes. (a) Primitive PVDF membrane, MP70; (b) PAA/PVDF composite membrane, grafting yield 0.39 mg/cm^2; (c) PAA/PVDF composite membrane, grafting yield 0.64 mg/cm^2.

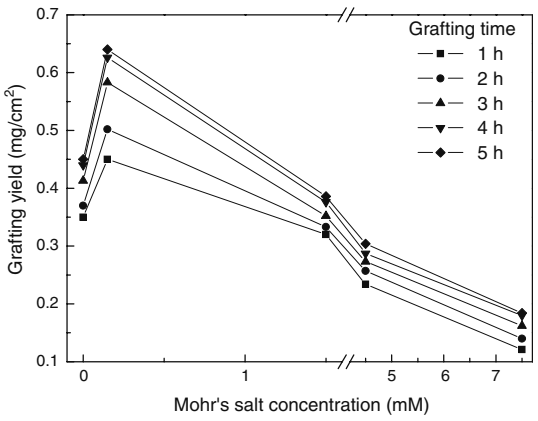


Fig. 3. Effect of Mohr's salt concentration (1.5×10^{-5}–7.5×10^{-3} M) on the grafting yield of PAA. Substrate: membrane 'MP70'; Plasma power: 100 W; Plasma irradiation time: 60 s.

mixture gelled after a certain period of time. For an already gelled sample, quantification of the grafting yield on the membrane was inaccurate, and thus the data are not reported herein. The effect of Mohr's salt on the grafting yield is clearly demonstrated in Fig. 3. The highest grafting yield was achieved when the salt concentration was 1.5×10^{-4} M. At lower or higher salt concentration, a smaller grafting yield results. This phenomenon is associated with the fact that free radicals (–CO·) produced by oxidation of ferrous ion can also be reduced to $-CO^{-}$, as pointed out by Chen et al. [12]

The grafting yield is also affected by the surface morphology of the substrate. As shown in Fig. 4, membrane 'MP70' has much higher grafting yields than membrane 'MS' for all grafting times. This is because porous surface has a larger area for radical generation and more void space for accommodation of growing polymer chains. The highest grafting yield for membrane 'MP70' reaches up to 0.64 mg/cm^2, whereas that for 'MW' was only 0.43 mg/cm^2. The same tendency has been reported previously [5]. Fig. 5 shows the SEM image of the composite membrane PAA/MP70 with a grafting yield of 0.64 mg/cm^2. Compared with Fig. 1(b), the primitive 'MP70', it can be seen that the small pores on the globular surface are now covered with a layer of PAA, which makes the surfaces of the globules appear dense and smooth. This PAA layer functions as an inter-linkage for subsequent l-lysine immobilization.

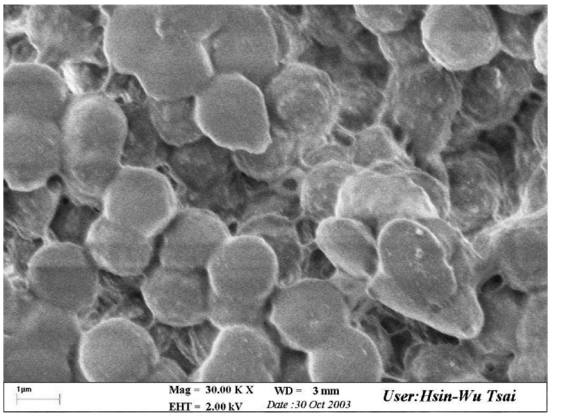


Fig. 5. SEM photomicrograph of membrane MP70 after being grafted with 0.64 mg/cm^2 of PAA.

Fig. 6 shows the effect of plasma treatment time on the grafting yield. For different reaction times, the grafting yields increase with increasing plasma irradiation time at first, and then decrease after passing through a maximum. The

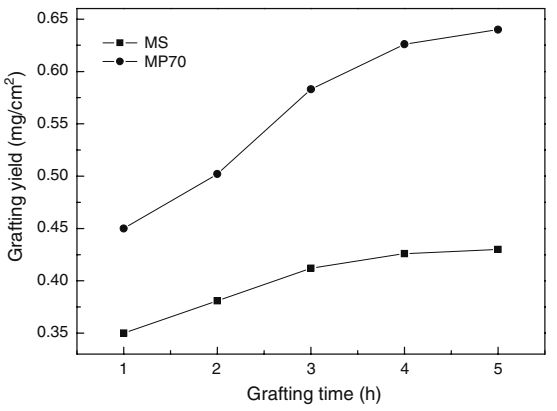


Fig. 4. Effect of surface morphology on the grafting yield of PAA. MS: nonporous surface; MP70: porous surface.

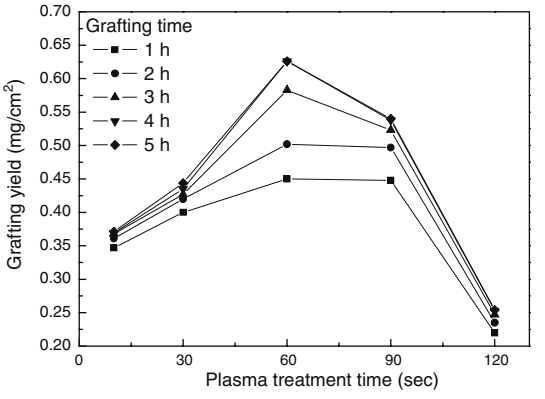


Fig. 6. Effect of plasma treatment time (10–120 s) on the grafting yield of PAA on membrane 'MP70'. The concentration of the Mohr's salt was 1.5×10^{-4} M, and the plasma power was 100 W.

highest yields all occur at a plasma-treatment time of 60 s. When the treatment time was too short, only a small amount of radicals was initially generated, leading a low grafting yield. However, if the membrane was subjected to excessive plasma bombardment, the grafting yield decreased as well. This is because membrane's surface structure might be changed by prolonged etching action of plasma, as has been pointed out by Lee et al. [13]. In a similar manner, the plasma power could also affect the grafting yield. Some typical results are shown in Fig. 7. The membrane substrate was 'MP70' and the plasma treatment time was 60 s. Apparently, the highest grafting yield was attained at the power 100 W for various grafting times. At a lower plasma power, not enough radicals were produced, whereas at a higher power, etching action took effect.

3.2. Immobilization of l-lysine on PAA/PVDF composite membranes

Chemical immobilization of l-lysine on the PAA-grafted membrane has been accomplished by forming amide bond between amine group of l-lysine and carboxylic acid of PAA. Because the reactions were carried out at pH 10, it was expected that the α-amine would dominate the amidation reaction by nuclear-philic substitution, whereas most γ-amine would survive intact throughout the immobilization process. This is due to the fact that at pH 10, α-amine is in the form of NH_2, and γ-amine is in the positively charged form of NH_3^+ [7]. The effects of membrane morphology, amount of PAA on the surface, and EDC activation conditions on the immobilization yield have been studied, and the results are discussed below.

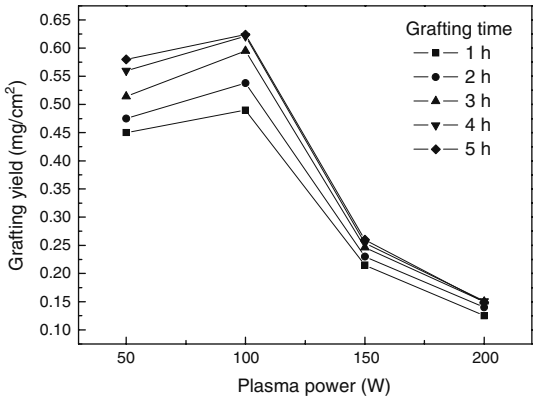


Fig. 7. Effect of plasma power (50–200 W) on the grafting yield of PAA on membrane 'MP70'. The concentration of the Mohr's salt was 1.5×10^{-4} M, and the plasma treatment time was 60 s.

3.2.1. Effect of PAA activation on l-lysine immobilization

Prior to immobilization, the grafted PAA has to be activated by EDC. This reaction, cf. Scheme 1, is known to affect the immobilization yield significantly. In this research, several relevant parameters were studied including activation time, pH, and the concentration of EDC, and the results are demonstrated in Fig. 8. Fig. 8(a) shows the amount of immobilized l-lysine vs. EDC concentration. The activation reactions were carried out at pH 2 for 5 h. It can be seen that the yield increases initially with increasing EDC concentration, then it levels off quickly when EDC concentration exceeds 0.02 M, and finally a constant value of 0.51 μg/cm^2 is achieved for EDC concentration higher than 0.03 M. This suggests that with only a small dosage of EDC, activation can reach a saturated level, beyond which immobilization yield will not increase even with a substantial increase of EDC concentration.

The effect of activation time on immobilization yield is shown in Fig. 8(b). The reaction was conducted at pH 3 with an EDC concentration of 0.03 M. The immobilization yield arrives at a constant value of 0.65 μg/cm^2 after 3 h of reaction. In other words, longer activation time would not result in higher immobilization yield as long as activation has attained a saturation level. It is beneficial to conduct the activation reaction in acidic conditions, only under which

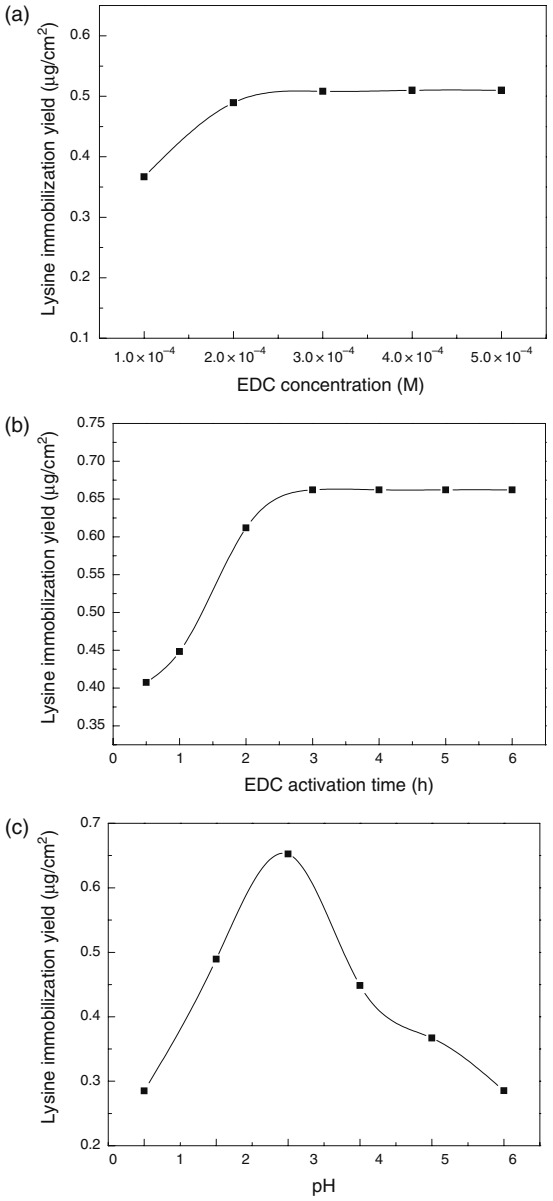


Fig. 8. Dependence of immobilization yield of lysine on (a) concentration of EDC; (b) EDC treatment time; (c) pH value of activation. The membrane was 'MP40' grafted with 0.6 mg/cm^2 of PAA.

o-acrylisouurea derivatives can be formed for subsequent reactions with l-lysine. Fig. 8(c) demonstrates the effect of acidity value over the range of pH 1–6. The activation time was 5 h and the concentration of EDC was 0.03 M. It can be seen that the immobilization yield increases first and then decreases again with a maximum yield occurring at pH 3, consistent with the result for heparin-immobilization shown previously [5] (Table 1).

Table 1
Preparation conditions of PVDF membranes for grafting PAA and immobilizing l-lysine

Membrane code	Coagulation bath	Surface structure		Tensile strength (Mpa)
		Top	Bottom	
MS	Water	Dense	Porous	1.77
MP40	40% DMSO	Dense	Porous	1.57
MP70	70% DMSO	Porous	Porous	1.22

Dope: PVDF dissolved in DMSO to form a 18% solution.

3.2.2. Effect of PAA amount on l-lysine immobilization

Membranes (both 'MP40' and 'MS') grafted with different amounts of PAA were immobilized with l-lysine to see the effects of PAA amount on the immobilization yield. The results are summarized in Table 2. For all cases, PAA activation was carried out at pH 3 for 5 h with an EDC concentration of 0.03 M. From Table 2, it can be seen that the amount of l-lysine is higher for membranes with larger amount of grafted PAA. Obviously, this is because more PAA offers more carboxylic acid for amidization with the amine groups of l-lysine. Furthermore, for the same amount of PAA, the porous membrane has a higher immobilization yield than the dense one. This is probably associated with the activation and/or immobilization wherein large surface area for chemical contact can be advantageous. However, the real causes are still unknown to us.

Table 2
Effect of membrane morphology on immobilization yield

Code	PVDF membrane substrate	Graft yield of PAA (mg/cm^2)	Immobilization yield of lysine ($\mu g/cm^2$)
MSL	MS	0.2	0.1
MSH	MS	0.4	0.2
MP40L	MP40	0.4	0.4
MP40H	MP40	0.6	0.65

3.3. Culture of PC12 cells on modified PVDF membranes

The MTT reduction activity of PC12 cells on the modified PVDF membranes and TCPS after 4 h, 1 and 2 days of incubation is shown in Fig. 9. The MTT assay relies on the ability of the viable cells to reduce a water-soluble yellow dye to a water-insoluble purple formazan product. The MTT value thus obtained is directly proportional to the cell number in each well. After cultured for 4 h, immobilized porous membranes show slightly better cell adhesion than do dense membranes. This suggests that cell adhesion may increase as the contact area increases. In addition, the MTT reduction activity of PC12 cells increases with increasing l-lysine content on the membrane surface after 4 h in culture. This is consistent with our previous study that l-lysine molecules immobilized on the substrate can promote neuronal MTT reduction activity [7]. Therefore, PC12 cells proliferating preferentially on membranes with larger amounts of l-lysine is predictable. The trend of the MTT reduction activity becomes more evident for longer culture periods. After 2 days of incubation, the MTT conversion of cells attached to the immobilized dense membrane is much less than those attached to the immobilized porous membranes. That is, the amount of l-lysine on the membrane for culturing PC12 cells is closely related to the cell activity. It is noted that the porous membrane with the maximal immobilization yield, 0.65 $\mu g/cm^2$ of l-lysine, gives rise to the highest formazan absorbance, even higher than that of the TCPS. Therefore, those membranes with high immobilization yields are considered to be more favorable for culture of neural cells, which also suggests using porous surface for modification to be a right choice.

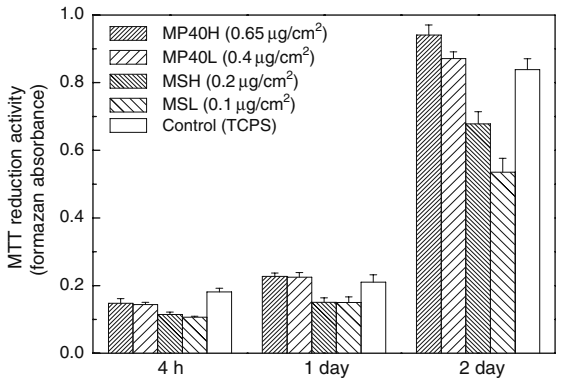


Fig. 9. MTT assay for different PVDF membranes grafted with l-lysine. Values are means ± standard deviation, $n = 4–6$.

4. Conclusion

In this research, l-lysine was covalently immobilized on both dense and porous PVDF membranes to create surfaces that are suited to neural culture. The immobilization yields of l-lysine were found to depend on the factors summarized below. (1) Larger amount of PAA initially grafted on PVDF results in higher immobilization yield. (2) The longer the activation time of PAA by EDC, the higher the immobilization yield. However, a saturated value was reached after 3 h of activation reaction. (3) The immobilization yield increased with increasing initial EDC concentration, yet a plateau value was reached when the concentration exceeded 0.03 M. (4) It is important to activate EDC in acidic conditions, and a maximum immobilization yield was obtained at pH 3.

(5) Porous membranes have higher PAA grafting and l-lysine immobilization yields than dense membranes, because the former have higher surface areas. It is also found that membranes with higher l-lysine contents have higher PC12 cell activity.

Acknowledgement

This research was supported by the National Council of Taiwan through grant NSC93-2216-E-032-002.

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