A pH-sensitive EVAL membrane by blending with PAA

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Abstract

The purpose of this study was to produce pH-sensitive domain in the dense poly(ethylene-co-vinyl-alcohol) (EVAL) membrane for colonic delivery of anti-cancer drug 5-fluorouracil (5-FU) by blending with a small amount of poly(acrylic acid) (PAA). The Fourier transform infrared (FTIR) spectroscopy analysis shows that PAA and EVAL were not very compatible in the PAA/EVAL blended membrane, whereas the intensity of self-association of both polymers was higher than that of inter-association between PAA and EVAL. It was proposed that PAA molecules would aggregate to act as the access for the transport of 5-FU through the blended membranes. The aggregated PAA gel phases were beyond the observable sensitivity of the scanning electron microscopy (SEM) and were low permeable for 5-FU at low pH, but significantly improved the permeability of 5-FU by the increase of pH of the medium, which agreed with the application of colon-specific drug delivery. When the membrane preparation temperature was changed from 60 to 45 °C, PAA domain contained in the blended membrane did not affect the permeation rate of 5-FU at different pH. This is because the membrane prepared at 45 °C was not so dense as that prepared at 60 °C. Therefore, the pH-sensitive characteristic of PAA domains would be applicable only to the membrane with a dense structure. Finally, it was demonstrated that 5-FU separated by the blended membranes still exhibited cytotoxicity toward Caco-2 cells at pH 7.4. Thus, the PAA/EVAL blended membrane is a potential material for the specific delivery of 5-FU to the colon for local treatment of colorectal cancer in the future clinical application.

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1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies in industrialized countries. Mortality of CRC is attributable to metastatic disease that occurs most often in the liver, followed by the lung. 5-Fluorouracil (5-FU) is one of the most widely used agents in the first-line chemotherapy of CRC [1–4], but produces severe hematological, mucosal and gastrointestinal toxicity, which is often encountered with dose intensification strategies and with moderate doses in adjuvant therapy treatment [5].

An understanding of 5-FU mechanisms of action has resulted in major therapeutic advances during the past decade; however, a plateau has been reached in the efficacy of 5-FU [6]. Maximizing therapeutic response by increasing selectivity is a major goal in the development of anticancer therapy. It would be highly beneficial to target 5-FU to a particular site within the gastroin-

testinal tract, either to maximize a therapeutic response or to reduce side effects caused by drug delivery to an inopportune region of the gut. The development of oral administration of 5-FU derivatives is one vigorously pursued direction without incomplete and unpredictable absorption due to its degradation in the gastrointestinal tract [7].

Within recent years, the development of drug delivery systems capable of selective release of drug in the colon has received much attention [8–10]. The optimum oral drug delivery system in such a case requires technologies that protect the drug through the “acidic” stomach and then distribute the drug to act topically in the “neutral” colon. Hydrogels have been used widely for the preparation of drug delivery systems with physically or chemically modulated responses. Generally, the pH-sensitive hydrogels are based on anionic polymers such as acrylic or methacrylic acids, which are water-impermeable at low pH, but the water can freely enter the swollen gel to act as a transport agent for drug by the change of pH of the medium [11–16].

Clinically, poly(ethylene-co-vinyl-alcohol) (EVAL) was used in hemodialysis with appropriate biocompatibility [17]. In our laboratories, EVAL membranes have been studied inten-

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sively over the past 10 years for different biomedical applications such as plasma protein separation [18], drug delivery [15,19] and neuron cell culture [20–22]. In the present work, poly(acrylic acid) (PAA)/EVAL blended membranes were prepared by the dry-cast process from mixed polymer solutions in a common solvent [23] and the permeation of 5-FU through the membranes were investigated at pH 2.0 and 7.4 for the development of pH-sensitive membranes. The present study is concerned with investigation of blends of EVAL with PAA in different ratios and prepared at different solvent evaporation temperature. The properties of PAA/EVAL blended membranes were investigated by scanning electron microscopy (SEM), Fourier transform infrared (FTIR) spectroscopy and water swelling analysis. We demonstrated that the PAA/EVAL blended membrane with 3.3 wt.% of PAA prepared at 60 °C was a potential material to reach a goal of releasing 5-FU for local treatment of CRC in the future clinical application.

2. Materials and methods

2.1. Materials

EVAL (E105A, containing ca. 56 mol% vinyl alcohol) was kindly supplied by Kuraray (Japan) and used as received. PAA used in this study was purchased from Aldrich (USA) and it has a quoted molecular weight of viscosity (M_v) of 750,000 and T_g of 106 °C. Ethanol was purchased from Showa (Japan). Water was double distilled and deionized before use. 5-FU was purchased from Sigma (USA) and used as the model drug for colon-specific delivery.

2.2. Membrane preparation

PAA and EVAL were dissolved in a cosolvent containing 40 vol.% water and 60 vol.% ethanol [23] to form 2 and 15 wt.% polymer solutions at 60 °C, respectively. The two solutions were mixed together in different ratios to give clear homogeneous PAA/EVAL blended solutions. The blended solutions were dispersed uniformly on Teflon plates (ca. 120 μm) at 60 °C, and then were immediately placed in an air-circulated oven at 60 or 45 °C until the casting solution became the solid membranes. Subsequently, the membranes were removed from the Teflon plate and dried in vacuum for 24 h. PAA/EVAL blended membranes were prepared with four different ratios as listed in Table 1. These membranes with the same composition such as ‘P-1’ were named as P-1-60C and P-1-45C according to the membrane preparation temperature 60 and 45 °C, respectively. The thickness of the membrane was $15 \pm 1 \mu\text{m}$. The freeze-dried

membranes were gold coated and viewed with a SEM (S-800, Hitachi, Japan) at 20 kV.

2.3. FTIR spectroscopy

The PAA/EVAL blended membranes were analyzed by FTIR spectroscopy employing a Bio-Rad FTX3000 spectrophotometer to investigate intermolecular interactions between PAA and EVAL in the blended membranes. All spectra were obtained at room temperature with a nominal resolution of 4 cm^{-1} and were signal averaged over 64 scans.

2.4. Swelling behavior of the membrane

A piece of a known weight membrane was immersed in buffered solution at pH 2–6 and 7.4 at 37 °C. The swelling was followed and then the swollen membrane was removed from the buffer solution and weighed after the superfluous liquid was carefully wiped with tissue paper. The swelling equilibrium was established until no further weight increase was observed. The swelling ratio (S_w) was determined according to the following expression:

$$S_w = \frac{W_s - W_d}{W_d} \quad (1)$$

where W_s is the weight of the swollen membrane and W_d is the weight of the dried membrane. All the data were averages of three independent experiments.

2.5. 5-FU permeation across the membrane

Permeation by diffusion of 5-FU through the prepared membranes was studied at pH 2.0 and 7.4 at 37 °C with 5-FU concentration of 50 $\mu\text{g/ml}$. The permeation experiments were carried out using a two-chamber diffusion cell with a volume of 40 ml each. The membranes with an effective permeation area equal to 5.3 cm^2 were clipped between the chambers. Vigorous agitation ca. 600 rpm was employed using independently controlled magnetic stirrers for both chambers. Since the permeability measurement was performed under sufficient stirring, the diffusion resistance at the liquid-membrane interface was neglected [24].

One of the diffusion chambers was filled with buffer solution containing 5-FU as the donor side and the other chamber was filled with buffer solution only as the receptor side. The permeability of 5-FU through the prepared membrane was monitored by periodically removing 200 μl samples from the receptor side and refilled back to avoid errors arising from the resulting volume variation. The concentrations of 5-FU were analyzed by an UV spectrophotometer (Ultrospec 1000E, Pharmacia Biotech, Sweden) from the peak absorbance at 266 nm. Each experiment was repeated three times and the results were expressed as the mean of the three results.

2.6. Viability of Caco-2 cells

In vitro test was carried out to test the viability of human colon cancer cell line, Caco-2 cells, exposed to 5-FU but separated

Table 1
The composition of PAA/EVAL blended membranes

No. of PAA/EVAL blended membrane	PAA/EVAL (wt.%)
P-1-60C, P-1-45C	0/100
P-2-60C, P-2-45C	1.5/98.5
P-3-60C, P-3-45C	2.3/97.7
P-4-60C, P-4-45C	3.3/96.7

Fig. 1. A schematic diagram of 5-FU permeation experiment combined with cell culture in a two-chamber diffusion cell.

by the prepared membranes in the diffusion cell as described above. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). One of the diffusion chamber was filled with minimum essential medium (MEM) containing 20% fetal bovine serum (FBS, Gibco BRL Life Technologies, Paisley, UK) and 5-FU at pH 7.4 and the other chamber was filled with MEM containing 20% FBS, antibiotic/antimycotic (penicillin G sodium 100 U/ml, streptomycin 100 mg/ml, amphotericin B 0.25 mg/ml, Gibco BRL Life Technologies) and Caco-2 cells cultured as a monolayer at pH 7.4, as shown in Fig. 1. The viability of Caco-2 cells was evaluated by cellular ability to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, M-2128, Sigma, St. Louis, MO, USA) [25] after the 5-FU permeation for 24 h at 37 °C. Since mitochondrial dehydrogenases of viable cells cleave selectively to the tetrazolium ring, yielding blue/purple formazan crystals, the level of the reduction of MTT into formazan can reflect the level of cell viability. For the MTT assay, the culture medium was removed, and then cells were incubated with 0.1 ml of MTT (2 mg/ml in PBS) for 3 h at 37 °C. After incubation, the medium was aspirated and the formazan reaction products were dissolved by dimethyl sulfoxide (Aldrich, Milwaukee, WI, USA) in PBS and shaken for 15 min. The optical density of the formazan solution was read on an ELISA plate reader (ELx 800, BIO-TEK, Winooski, VT, USA) at 570 nm. Cell viability determined by the MTT assay was expressed as percentage of control Caco-2 cells without adding 5-FU into the diffusion chamber.

3. Results and discussion

3.1. Membrane morphology

Macroscopically, EVAL and PAA/EVAL blended membranes prepared at 60 and 45 °C appeared colorless transparent.

Fig. 2. The SEM photomicrographs of top surface and cross-section of: (a) P-1-60C and (b) P-1-45C membranes.

Microscopically, EVAL membranes prepared at 60 and 45 °C showed slight difference, as indicated in Fig. 2. The EVAL membrane prepared at 60 °C (P-1-60) appeared fairly dense structure in top surface and cross-section. The microscopic analysis of the membrane prepared at 45 °C (P-1-45) also showed no holes existing in the membrane surface, but its cross-section was not so dense as that of P-1-60 membrane. After EVAL blended with PAA, the blended membranes (P-2, P-3 and P-4) showed similar dense morphology to the pure EVAL membrane, regardless of membrane preparation temperature (not shown here). Thus, the blending processes did not change the membrane structure under the observable detection sensitivity of the SEM.

3.2. Interaction between PAA and EVAL in the blended membranes

To investigate the interaction between PAA and EVAL in the blended membranes, samples were analyzed by IR absorption spectroscopy. Fig. 3 shows the FTIR spectra in the 3000–3800 cm^{-1} region for the hydroxyl-stretching band of EVAL and PAA/EVAL blended membranes. This broad band is known to consist of contributions from hydroxyl groups surrounded by different environments: hydroxyl groups hydrogen-bonded with other hydroxyl groups in the same or different chains and non-hydrogen-bonded hydroxyl groups [26]. When the spectra of EVAL membrane was compared, the maximum of this band of the PAA/EVAL blended membranes shows a progressive shift from 3348 to 3358 cm^{-1} as the PAA con-

Fig. 3. FTIR spectra of the hydroxyl stretching region of: (a) P-1-60C, (b) P-2-60C, (c) P-3-60C and (d) P-4-60C membranes.

tent increased. This result suggests that a redistribution in the arrangement of the hydroxyl group associations of the EVAL, which either are involved in the association processes for pure EVAL or hydrogen-bonded to the hydroxyl groups in PAA. The shift toward a higher wave number side indicates that the intensity of the inter-association between PAA and EVAL in the blended membranes was lower than that of self-association in the pure EVAL membrane.

The FTIR spectra in the carbonyl stretching region for the EVAL and PAA/EVAL blended membranes are shown in Fig. 4. As the PAA was blended with EVAL, the carbonyl absorption band at around 1716.6 cm^{-1} appeared. The intensity of this band increased upon increasing PAA content in the blended membranes. The carbonyl absorption band of pure PAA is at 1711 cm^{-1} [27]. Since the PAA carbonyl group could be hydrogen-bonded to the EVAL hydroxyl group or the PAA carboxyl group, the absorption band shifting to a higher wave number side indicated that the hydrogen bonding between PAA and EVAL was relatively weak than that in pure PAA.

Based on Figs. 3 and 4, the interaction of hydrogen bonding between PAA and EVAL declined the intensity of self-association in pure PAA or in pure EVAL, but the intensity of self-association of both polymers was still higher than that of inter-association between PAA and EVAL. Therefore, PAA

Fig. 4. FTIR spectra of the carbonyl stretching region of: (a) P-1-60C, (b) P-2-60C, (c) P-3-60C and (d) P-4-60C membranes.

Fig. 5. Swelling ratio of EVAL and PAA/EVAL blended membranes prepared at 45°C in buffer solutions with different pH values at 37°C .

could be added into the EVAL membranes, but PAA and EVAL were not very compatible in the blended membrane. Actually, as the PAA content exceeded about 6%, the blended solution became so turbid that a homogeneous solution became mechanically impractical. For this reason, it is reasonable to assume that PAA molecules could segregate into distinct phases in the blended membranes. Although such modification by blending with a small amount of PAA could not be detected under SEM, it had an influence on different properties of the resulting membrane: swelling ratio and 5-FU permeability. This detail will be described below.

3.3. Swelling ratio of the membrane

Figs. 5 and 6 show the swelling ratio of EVAL and PAA/EVAL blended membranes prepared at 45 and 60°C , respectively, in buffer solutions with different pH values at 37°C . The swelling ratio was the maximum hydration degree

Fig. 6. Swelling ratio of EVAL and PAA/EVAL blended membranes prepared at 60°C in buffer solutions with different pH values at 37°C .

reached after immersion of three samples in an aqueous solution of a prescribed pH. The water swelling characteristic was examined because it had a significant influence on drug permeation across the membrane. The swelling ratio of the membrane may be mainly determined by three factors, i.e. membrane preparation temperature, PAA content of the membranes and pH of buffer solution. As shown in Figs. 5 and 6, the swelling ratio of membranes prepared at 45 °C was always greater than that of membranes prepared at 60 °C, which was consistent with the observation under SEM that membranes prepared at 45 °C was not so dense as those prepared at 60 °C. In addition, the swelling ratio of membranes increased with the increase of PAA content. This is because PAA favors the uptake of water into the PAA phase of membrane; thus, the introduction of PAA into the membranes increased membrane swelling ratio. More remarkable is the pH dependence of the membrane swelling. Besides the swelling ratio of pure EVAL membranes decreased slightly with the increase in pH, the swelling ratio of the blended membranes was greatly increased when the pH of buffer solution increased. This indicated that the PAA/EVAL blended membranes could provide different swelling ratio for drug delivery at different pH values.

3.4. 5-FU permeability across the membrane

Figs. 7 and 8 show the time dependence of the cumulative amount of 5-FU permeation through EVAL and PAA/EVAL blended membranes prepared at 45 and 60 °C, respectively, at pH 2.0 and 7.4. Basically, the permeation rate of 5-FU through all of the membranes increased with the increase of PAA content, which was consistent with the result that PAA could increase the swelling ratio of the blended membranes. When the data were evaluated based on membrane preparation temperature dependence, the permeation rate of 5-FU through the membranes prepared at 45 °C was always greater than those prepared at 60 °C, which was also consistent with the swelling measurement that membranes prepared at 45 °C had higher swelling degree.

When the data were evaluated based on the pH of buffer solution, there was no significant difference for the cumulative amount of 5-FU in the first 24 h at pH 2.0 and 7.4 (Fig. 7a and b) for membranes prepared at 45 °C. This indicates that PAA could increase the permeation rate of 5-FU through the blended membranes but could not further provide improved permeability of 5-FU in the acidic or neutral environment. Thus, PAA could not modify the EVAL membranes prepared at 45 °C to result in the conversion of the membrane to be pH-sensitive.

Now, 5-FU flux values are given to more clearly indicate the pH dependence of 5-FU permeability through the prepared membranes. Fig. 9 shows the 5-FU flux through EVAL and PAA/EVAL blended membranes at pH 2.0 and 7.4. The 5-FU flux (J) was obtained at time zero of Figs. 7 and 8 by using $J = V\Delta C/A\Delta t$, where V is the volume of diffusion chamber, ΔC the drug concentration difference across the membrane, A the membrane area and Δt is the diffusion time. There was no clear difference for the 5-FU flux through the membranes prepared at 45 °C between at pH 2.0 and 7.4 (Fig. 9a). Conversely, when membranes were prepared at 60 °C, the pH-sensitive effect

Fig. 7. Cumulative amount of 5-FU permeation through EVAL and PAA/EVAL blended membranes prepared at 45 °C at (a) pH 2.0 and (b) pH 7.4.

of PAA was significant, at each of the PAA/EVAL blended membranes studied (Fig. 9b). The 5-FU flux at pH 7.4 was much higher than that at pH 2.0. Therefore, there was much higher cumulative amounts of 5-FU permeation through the PAA/EVAL blended membranes at pH 7.4 relative to at pH 2.0 (Fig. 8a and b). Nearly, the cumulative amounts of 5-FU permeation through P-2-60C, P-3-60C and P-4-60C membranes after 24 h increased about 4.5-, 4.9- and 5.7-folds, respectively, for the pH value changed from 2.0 to 7.4. Such high cumulative amounts of 5-FU permeating across the PAA/EVAL blended membranes prepared at 60 °C actually were comparable with membranes prepared at 45 °C. This indicates that the PAA/EVAL blended membranes prepared at 60 °C could provide pH-selective permeability for 5-FU, which were low permeable for 5-FU at low pH, but significantly improved the permeability of 5-FU by the increase of pH of the medium. The cumulative amount of 5-FU permeation through the EVAL membranes prepared at 60 °C was conspicuously small at pH 2.0 and 7.4. It is believed that dense membrane structure and without the addition of PAA led to the low permeability of 5-FU.

Fig. 8. Cumulative amount of 5-FU permeation through EVAL and PAA/EVAL blended membranes prepared at 60 °C at (a) pH 2.0 and (b) pH 7.4.

Based on these experimental observations and the general assumptions, the rationale for the formation of pH-sensitive membranes from adding PAA into the EVAL membrane would then be as follows: although the interaction of hydrogen bonding between PAA and EVAL declined the intensity of self-association in both polymers, the intensity of self-association of both polymers was still higher than that of inter-association between PAA and EVAL. Therefore, when the blended membranes were prepared at 60 °C, PAA and EVAL molecules would separately tend to aggregate to form the dense structure by virtue of a great number of molecular entanglements. At this time, PAA domain could be considered as hydrogel phase [28]. It is known a hydrogel can be characterized by its capacity to adsorb water. The water content in the equilibrium swelling affects the solute permeability across the hydrogel. Thus, it is reasonable to suggest most of 5-FU permeated through the PAA/EVAL blended membranes by way of water-swollen region of the PAA domain because the cumulative amount of 5-FU permeation through the pure EVAL membrane at pH 2.0 and at pH 7.4 appeared to be virtually the same and relatively low. In addition, the carboxylic acids of PAA can dissociate to carboxylate ions at pH 7.4 to provide high charge density in the PAA domain. Therefore, the greater permeation rate of 5-FU at pH 7.4 rather than at pH 2.0

Fig. 9. Flux of 5-FU through EVAL and PAA/EVAL blended membranes prepared at (a) 45 and (b) 60 °C at pH 2.0 and 7.4.

was due to the electrical repulsion between adjacent carboxylate ions and a transition of compact coiled PAA chains to extended ones to swell PAA domain. Consequently, PAA/EVAL blended membranes may be considered as PAA hydrogel domains dispersed within the EVAL matrix and the mesh size of hydrogel phase is tight and compact at pH 2.0 but is loose and diluted at pH 7.4 [28], namely pH-sensitive characteristic in the EVAL membrane was successfully created by blending with PAA. Furthermore, the fluorescein isothiocyanate-dextran with average molecular weight 40 K was used as the feed to evaluate the pH-sensitive mesh size. The P-4-60C membrane was found to reject dextran at pH 7.4. This suggests that the available space for drug permeation through P-4-60C membrane was less than the size of dextran with average molecular weight 40 K. Therefore, it is not surprised that these pH-sensitive phases were beyond the observable sensitivity of the SEM and acted as the access for the transport of nano-scale solute through the blended membranes.

On the other hand, PAA domain contained in the blended membrane prepared at 45 °C did not affect the permeation rate of 5-FU at different pH values. This is because the membranes themselves were not so dense that even the swelling ratio of P-1-45C membranes at pH 2.0 was very close to that of P-4-60C membrane prepared at pH 7.4 (Fig. 6). The other possible origin of the enhancement of 5-FU permeability across the PAA/EVAL

Fig. 10. Viability of Caco-2 cells exposed to 5-FU, separated by the P-4-60C membrane in the diffusion cell at pH 7.4 for 24 h.

blended membranes prepared at 60 °C was that the 5-FU partition in the membrane increased for the pH value changed from 2.0 to 7.4. However, the blended process in this study did not significantly change the permeation of 5-FU for the membranes prepared at 45 °C. Therefore, the enhancement of 5-FU permeability in the blended membranes at pH 7.4 could not be ascribed to the higher partition coefficient of 5-FU in the PAA hydrogel phases. Consequently, the pH-sensitive characteristic of PAA domains would be applicable only to the membrane exhibit a relatively dense structure.

3.5. Viability of Caco-2 cells

In order to simulate the *in vivo* condition, Caco-2 cells and 5-FU were separated by the P-4-60C membrane in the diffusion cell at pH 7.4. The toxicity of 5-FU permeating through the membrane toward Caco-2 cells was evaluated by the MTT assay following 24 h of permeation at 37 °C. Compared to control cells, cell viability was dramatically decreased to $36 \pm 3.4\%$ in the presence of permeated 5-FU (Fig. 10). This finding indicated that Caco-2 cells were indeed damaged for 5-FU permeation though the P-4-60C membrane at pH 7.4. Therefore, such a diffusion cell combining with cell culture can be extensively applied to other studies for drug delivery *in vitro*.

4. Conclusion

In the present study, the non-pH-sensitive EVAL membrane prepared at 60 °C could be converted to pH-sensitive by adding a small amount of PAA. According to the FTIR spectra and swelling measurements, it is suggested that PAA domains dispersed within the EVAL matrix and PAA plays an important role on the pH-sensitivity of the blended membranes. The study also revealed that 5-FU separated by the blended membranes still exhibited cytotoxicity toward Caco-2 cells at pH 7.4. Thus, the PAA/EVAL blended membrane is a potential material for the specific delivery of 5-FU to the colon for local treatment of CRC in the future clinical application.

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