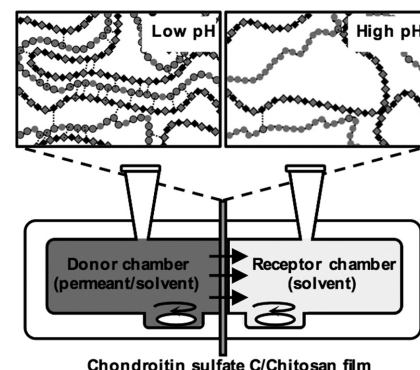# Kinetic Analysis of Molecular Permeabilities of Free-Standing Polysaccharide Composite Films

Kazutoshi Iijima, Takayuki Kimura, Ryo Sato, Tomoki Takahashi, Mineo Hashizume*

This study is performed in order to assess the molecular permeabilities of composite films of chondroitin sulfate C (CS) and chitosan (CHI) prepared by the hot press technique. Permeation behaviors are evaluated using horizontal diffusion cells and four substrates with different charges: methylene blue (MB), orange II (OR), L-tryptophan (Trp), and 4-methylumbelliferyl β-D-galactopyranoside (MUG). Permeability coefficients, diffusion coefficients, and partition coefficients are calculated from permeation curves. When MB and OR are used as the permeants, permeability coefficients in solutions with higher pH or ionic strength are greater than in those with lower pH or ionic strength. Permeability coefficients at pH 5.9 at which Trp carries zero net charge are as follows: Trp > MB ≈ MUG > OR. The diffusion coefficients and partition coefficients obtained indicate that CS/CHI films permeate these molecules based on a partition mechanism at lower pH and a pore model at higher pH. These characteristics appear to reflect the unique microenvironment of films consisting of polyion complexes of oppositely charged polysaccharides.

Low pH · High pH · Donor chamber (permeant/solvent) · Receptor chamber (solvent) · Chondroitin sulfate C/Chitosan film

1. Introduction

Polyion complexes (PICs) formed from oppositely charged natural and/or synthesized polymers by electrostatic interactions have a wide range of applications from industrial uses such as pervaporation separators,[1] gas separators,[2] and sensors[3] to biomedical uses including drug carriers,[4] dressing materials,[5] and scaffolds for tissue engineering.[6] Various forms of PICs such as nano/micro particles and capsules, films, fibers, and hydrogels may be prepared without the use of covalent cross linkers or condensing agents. For example, the layer-by-layer (LbL) assembly technique, in which substrates are alternately immersed in oppositely charged polyelectrolyte solutions, is useful for preparing PIC films and coating substrates with PIC layers. LbL films with various compositions such as those of chitosan (CHI) with dextran sulfate,[7] hyaluronic acid,[8] heparin,[9] and alginate[10] have been readily obtained and applied as stimulus-responsive drug carriers.[11] Relatively thick films may also be prepared using this technique by repeating the assemblies a large number of times.[10]

We developed polysaccharide composite films made of anionic polysaccharides such as chondroitin sulfate C (CS),

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hyaluronic acid, and heparin, and the cationic poly-saccharide chitosan using the hot press technique.[12,13] In this method, free-standing transparent films with thick-nesses of several tenths of micrometers were obtained in less than 30 min by hot pressing PIC gels.[13] We also investigated the potential use of composite films made of CS and CHI (hereafter denoted as CS/CHI films) as drug carriers.[14] Thicker films load larger amounts of drugs when they are used as drug carriers. Cationic methylene blue (MB) and anionic carboxy fluorescein (CF) may both be incorporated into CS/CHI films by the addition of these molecules into solution during PIC formation or soaking films in solutions containing these molecules. CS/CHI films have been reported to exhibit medium-responsive release behaviors.[14] The release rates of anionic CF were previously found to be higher than those of cationic MB, and release rates were faster at higher pH than at lower pH for MB and CF. Releasing behaviors in solutions with various pH were similar to the degree of swelling of films in these solutions, whereas a clear correlation between the time course of swelling and that of releasing was not observed.[14]

Permeability is also an essential factor influencing the characteristics of film materials. Permeabilities of flat films of CHI,[15,16] CHI/polyhedral oligosilsesquiox-anes,[17] collagen,[18] polyurethane,[19] and hydroxyethyl methacrylate-based hydrogel[20] were investigated using various permeants, from low molecular weight (MW) com-pounds to proteins. The permeation of molecules through a film involves three steps: the dissolution of permeants in polymer films, diffusion along polymer chains within the films, and release from the films into the solution, and a detailed kinetic analysis of the permeation behaviors of films provides an insight into the partition and diffusion of molecules in films without the effects of initial swelling.[15] For example, detailed analysis of permeation behaviors of urea through polyurethane-derivatives films revealed that the permeability was strongly affected by hydrophobicity inside the films and these results gave the insights for designing polyurethane-coated granule drugs with desired releasing property.[19] In the present study, the molecular permeabilities of CS/CHI composite films were analyzed kinetically using four kinds of permeants with almost the same sizes, but different charges. Another aim of this study was to evaluate CS/CHI films as separation membranes by examining their permeant selectivities.

2. Experimental Section

2.1. Materials

CS (sodium salt, from shark cartilage, MW ≈ 20 000), CHI (from crab shell, MW > 100 000), p-(2-hydroxy-1-naphthylazo)benzene-sulfonic acid sodium salt (orange II, OR), and L-tryptophan (Trp)

were purchased from Nacalai Tesque Inc. MB and 4-methyl-umbelliferyl β-D-galactopyranoside (MUG) were obtained from Kanto Chemical Co., Inc. and Wako Pure Chemical Industries, Ltd., respectively. All chemicals were used without further puri-fication. Cellulose dialysis tubes (thickness of 40 µm, MW cutoff (MWCO): 12 000–14 000) were purchased from Nihon Medical Sci-ence, Inc. The distilled water and ultrapure water (18.2 MΩ cm) used in experiments were prepared using Advantec RFD210TA and Advantec RFU414BA, respectively.

2.2. Preparation of Polysaccharide Composite Films

Polysaccharide composite films made of CS and CHI were pre-pared according to our previous study.[13] Briefly, 2 wt% aqueous solutions of CS were added drop wise to 1 wt% CHI solution in 1 wt% aqueous acetic acid until the formation of gel-like PICs was completed. After washing with ultrapure water, these PICs were collected by centrifugation (6000 rpm, 5 min). The PIC gel was sandwiched with polytetrafluoroethylene (PTFE) sheets and polyethylene terephthalate (PET) sheets, which were pressed using a hot press apparatus (AH-2003; AS ONE Corp.) preheated to 120 °C at 20 MPa. Pressure was immediately decreased to 0 MPa and the spread film-like PIC gel was removed from the hot press stage. A small amount of ultrapure water was applied to the film-like PIC gel and left for ≈10 min in order to swell the PIC gel. The swollen PIC gel was sandwiched with PTFE and PET sheets and hot pressed for 10 s at 20 MPa. The film-like PIC gel was folded and hot pressed for 3 min at 20 MPa with a PET spacer having a rectangular hole (5 cm × 5 cm). The edges of the film were then trimmed using a cutter knife.

2.3. Measurement of Film Permeabilities

Diffusion cells (Side-Bi-Side cells; PermeGear, Inc.) were used in all permeation studies. A schematic illustration of the apparatus is shown in Figure 1A. A 0.64 cm² area was exposed to the donor and receptor compartments of each diffusion cell, and each com-partment had a volume of 3.0 mL and was magnetically stirred. CS/CHI films were cut into 1.5 cm × 1.5 cm squares, swelled in solvent for 15 min, and then mounted between the donor and receptor compartments. The donor cell was filled with 3.0 mL of permeant solution (4×10^{-4} M in ultrapure water, phosphate buffer (PB; 10×10^{-3} M and 100×10^{-3} M of phosphate ion spe-cies, pH 5.8, 7.4, and 8.0) and phosphate-buffered saline (PBS; 10×10^{-3} M of phosphate ion species and 137×10^{-3} M of NaCl, pH 7.4), and the receptor cell was filled with 3.0 mL of the same solu-tion without the permeant. This point was set to time zero. After the predetermined times, 50 µL of the solution in the receptor compartment was collected and diluted 20-fold by the solvent. UV–visible spectra of the solutions were obtained using a spec-trophotometer (V-660, JASCO Corp.) at 200 nm min⁻¹ between 200 and 800 nm. Absorbances at 665, 486, 218, and 315 nm were used to calculate the amounts of MB, OR, Trp, and MUG, respec-tively, which were plotted as a function of time. The kinetic coef-ficients of permeation were calculated using Equations (1)–(3)[15]

$$J = \frac{\mathrm{d}M}{S \cdot \mathrm{d}t} \approx \frac{D \cdot K \cdot C_1}{l} \approx P \cdot C_1 \qquad (1)$$

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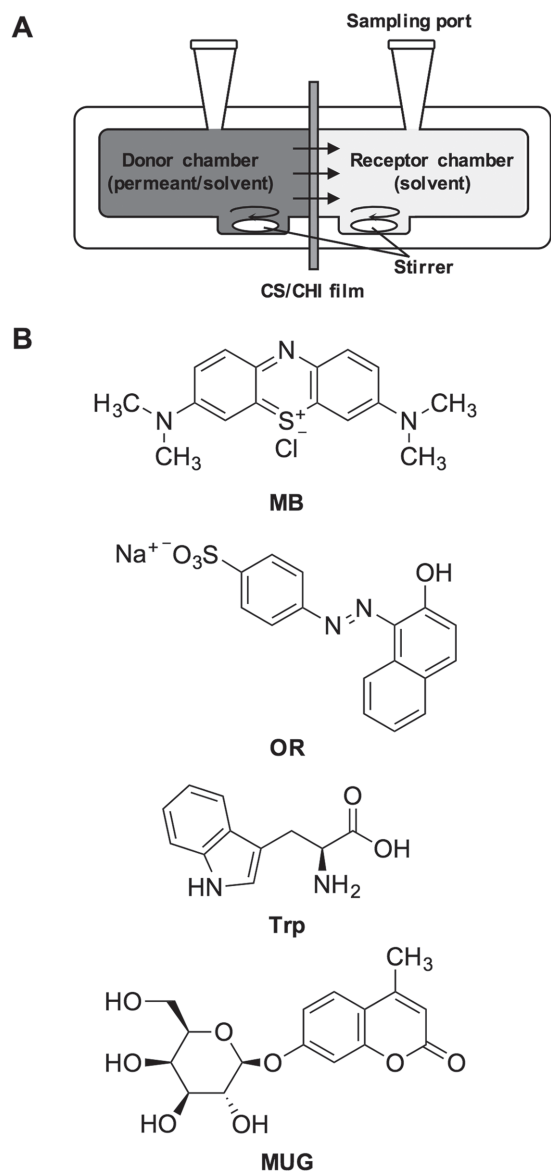© 2016 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

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Figure 1. A) Schematic view of the horizontal diffusion apparatus used in this study. B) Chemical structures and abbreviations of permeants used in this study.

$$D = \frac{l^2}{6\theta} \tag{2}$$

$$K = \frac{P \cdot l}{D} \tag{3}$$

Here, J represents the flux, M represents the amount of the permeant, D represents the diffusion coefficient, and P represents the permeability coefficient. S and l mean the permeation area and thickness of the swollen film, respectively. C_1 means the initial concentration of the permeant. The partition coefficient K was defined as the ratio of the permeant concentration in the membrane to that in the bulk solution. θ is the time lag measured from the intercept of the permeation graph.

2.4. Film-Swelling Behaviors

The swelling behaviors of the films were evaluated according to previously described methods.[13,14,21] Films (1 cm × 1 cm) were immersed in ultrapure water and other buffer solutions and incubated at room temperature for 30 min. The degree of swelling (%) was calculated using Equation (4)

$$\text{Degree of swelling}\,(\%) = \frac{W_s - W_I}{W_I}\,100 \tag{4}$$

In this equation, W_I represents the initial weight of the film before its immersion and W_s represents the weight of the swollen film after its immersion.

3. Results and Discussion

3.1. Permeation Behaviors of MB in CS/CHI Films and a Dialysis Membrane

CS/CHI films with a thickness of ≈100 μm under dry conditions were prepared according to our previous study.[13] The film was yellow, transparent, and free-standing and became soft and flexible after becoming swollen in solution. The thickness of the swollen film was ≈150 μm and used in the analysis. Two mechanisms have been proposed for the permeation of molecules through films.[20] One is the pore mechanism, in which permeants are transported through pores or channels in films and the representative is a dialysis membrane.[22] The other mechanism is the partition mechanism, in which permeants are dissolved and diffused within a film and the representative is a lipid bilayer.[23] The permeation behaviors of CS/CHI films using the monocationic dye MB (Figure 1B) in solution with different pH, ultrapure water, and 100×10^{-3} M PB (pH 5.8, 7.4, and 8.0) were investigated and compared with a 40 μm thick cellulose dialysis membrane with MWCO of 12 000–14 000. Typical curves of the concentration of MB in the receptor compartments of CS/CHI films (Figure 2A) and the dialysis membrane (Figure 2B) were shown. Permeability coefficients P were then calculated from the permeation curves using Equation (1) (Figure 2C).

The permeability coefficient is the amount of a permeant that passes through a unit area of a film per unit time. In the case of the dialysis membrane, permeability coefficients were similar under PB with different pH, except for ultrapure water, which showed the highest value (Figure 2C). The size of pores in the dialysis membrane did not appear to change under different pH and resulted in constant permeability coefficients. This is consistent with the pore model of the dialysis membrane. In contrast, the permeability coefficients of CS/CHI films in PB were markedly higher than those in ultrapure water. Among PB, the permeability coefficients of CS/CHI films in pH 7.4 and 8.0 were higher than that in pH 5.8, and
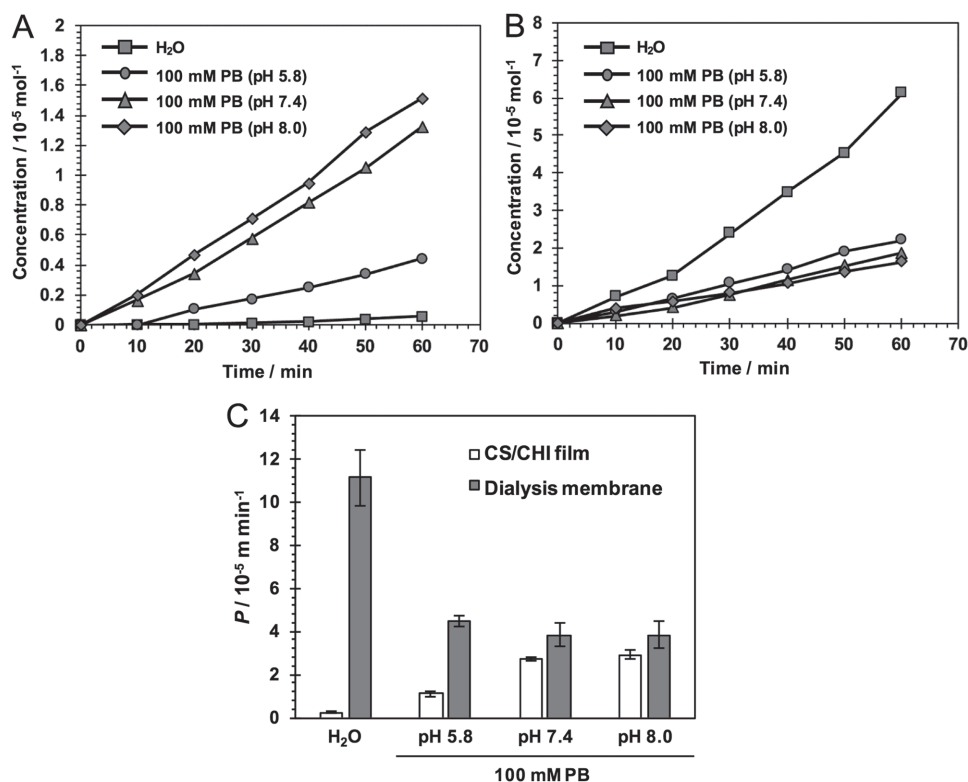
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Figure 2. Permeation behaviors of CS/CHI films and the dialysis membrane using methylene blue (MB) as a permeant. Permeating behaviors of A) CS/CHI films and B) the dialysis membrane in ultrapure water and 100×10^{-3} M phosphate buffer (PB) with different pH, and C) permeation coefficient P.

similar to that of the thinner dialysis membrane. CS/CHI films after the permeation experiment were stained with MB (Figure S1, Supporting Information), and the partition of MB in the films was suggested. These results indicate that CS/CHI films permeate molecules by the partition mechanism and/or pore mechanism, in which pore size changed depending on the pH of the solvent.

3.2. Kinetic Analysis of Permeation Behaviors of Monocationic and Monoanionic Dyes in CS/CHI Films

The permeation behaviors of CS/CHI films in solutions with different pH and ionic strengths were investigated using monocationic MB and monoanionic OR (Figure 2B) in ultrapure water, 10×10^{-3} M PB (pH 5.8, 7.4, and 8.0), and PBS (10×10^{-3} M of phosphate ion species and 137×10^{-3} M of NaCl, pH 7.4). CS/CHI composite films were stable in the solutions used in the permeation studies during the experiments (Table S1, Supporting Information). Typical curves of the concentration of MB (Figure 3A) and OR (Figure 3B) in receptor compartments were shown. In addition to permeability coefficients P, diffusion coefficients D and partition coefficients K were calculated from permeation curves using Equations (2) and (3)[15] assuming the partition model. These results are summarized in Figure 3C–E.

At pH 5.8, the permeability coefficient of MB was higher than that of OR (Figure 3C). In MB and OR, permeability coefficients in higher pH (7.4 and 8.0) were markedly greater than that in lower pH (pH 5.8; Figure 3C). Comparisons between 10×10^{-3} M PB (pH 7.4, ionic strength $I = 0.02$; ionic strength I is a function of the concentration of all ions present in the solution calculated as Equation (5)[24]) and PBS (pH 7.4, 10×10^{-3} M of phosphate ion species and 137×10^{-3} M of NaCl, $I = 0.16$) indicated that permeability was markedly greater in the solution with higher ionic strength than in that with lower ionic strength

$$I = \frac{1}{2}\sum_{i=1}^{n} c_i z_i^2 \tag{5}$$

Here, c_i is the molar concentration of the ion and z_i is the charge number of the ion.

The swelling behaviors of CS/CHI films in various solutions were evaluated because they may be affected by the pH and ion strength of solvents (Figure 3F). The order of the swelling ratio was as follows: PBS > PB (pH 8.0) > PB (pH 7.4) > PB (pH 5.8) ≈ ultrapure water, as we reported previously.[14] The swelling ratio correlated well with permeability coefficients (P) and releasing behaviors from MB-loaded films.
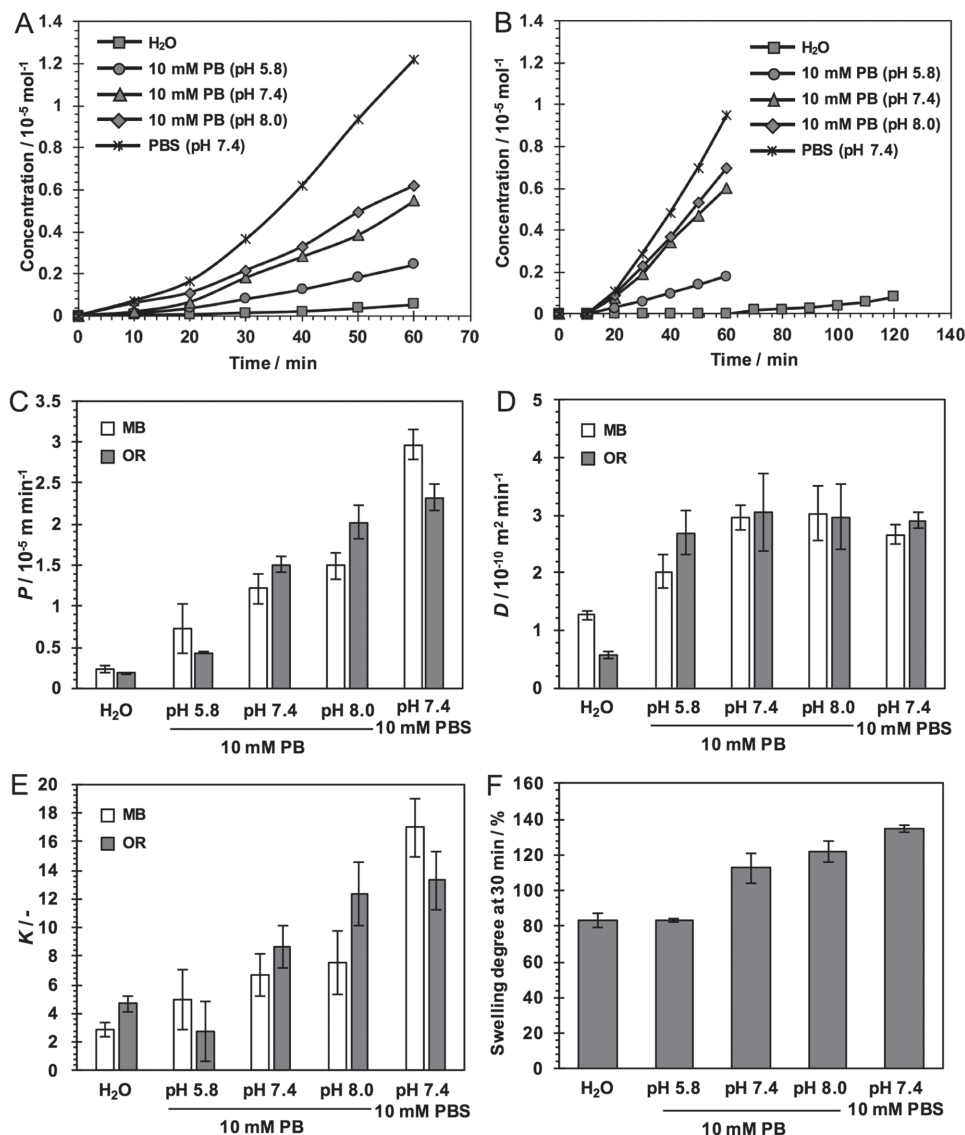
Figure 3. Permeation behaviors of CS/CHI films in ultrapure water, 10×10^{-3} M PB with different pH, and PBS (pH 7.4) using A) MB and B) orange II (OR) as permeants, C) permeability coefficients P, D) diffusion coefficients D, E) partition coefficients K, and F) degrees of swelling of CS/CHI films after 30 min incubation periods in different buffers.

Correlations also need to be considered among the swelling ratio of films, permeability through films, and release behaviors from dye-loaded films, with the latter two factors corresponding to D (Figure 3D) and K (Figure 3E), respectively. pH- and ionic strength-dependent permeation behaviors were also observed in capsules with synthetic polyelectrolyte multilayers prepared using the LbL assembly technique, and these were explained based on the charges of polyelectrolytes.[25] Since CS/CHI PICs were prepared by titration, namely, CS solutions were added to CHI solutions until PICs formed completely, the net charge of PICs was expected to be close to zero. Therefore, similar amounts of reactive cationic and anionic groups appeared to exist in as-prepared

CS/CHI films. However, this stoichiometry, the balance of cationic and anionic functional groups, may be altered by changes in the pH of the environmental condition because the pK_a values of the carboxylic acid groups of CS and ammonium ion groups in CHI were 3.8[26] and 6.5,[27] respectively. PICs were formed by mixing aqueous solutions of CS and CHI in 1 wt% aqueous acetic acid and the pH of the mixed solution was 4.3. Under this condition, most of the amino groups in CHI, the sulfate groups of N-acetyl-D-glucosamine 6-sulfate units in CS, and 75% of the carboxylic acid groups of glucuronic acid units in CS were ionized (Figure S2, Supporting Information), and the electrostatic interaction between them contributed to the crosslinking of CS and CHI (Figure 4A). By changing
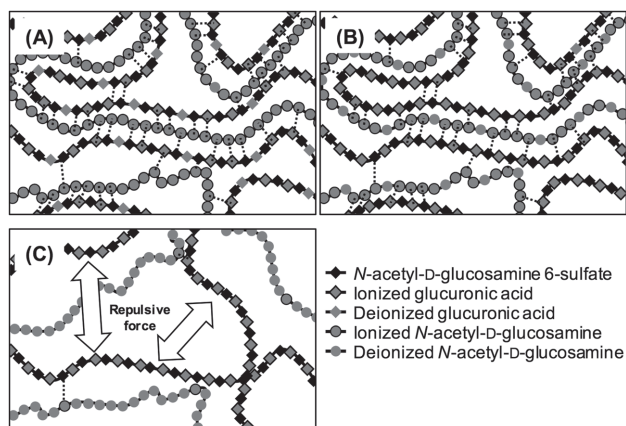
Figure 4. Schematic illustration of the internal structure of CS/CHI films in solutions with various pH. A) pH 4.3 (PIC formation), B) pH 5.8 and 5.9, and C) pH 7.4 and 8.0.

pH to 5.8, the deprotonation of carboxylic acid groups and ammonium ion groups proceeded. Most of the carboxylic acid groups were ionized, while 20% of the ammonium ion groups in CHI were deionized (Figure S2, Supporting Information). These changes made the inside of the films weakly anionic (Figure 4B). pH changes from 5.8 to 7.4 and 8.0 had more prominent effects. They decreased ammonium ion groups in CHI to less than 10%. On the other hand, the degree of dissociation of the sulfate and carboxylic acid groups was not significantly affected. Therefore, the number of ion complexes appeared to decrease in the films and repulsion forces were observed between CS molecules inside the films. This made films more swollen and anionic. A high ionic strength also reduced electrostatic interactions between polysaccharides and resulted in a decrease in the number of ion complexes.

These results indicate that swelling is not the driving force for the release of molecules from and their permeation through films; in other words, not significantly affect D values (Figure 3D). This may have been due to the permeant molecules used in this study being relatively small. When films swelled by more than a certain degree, the inner free volumes of the films may have become markedly larger than that affecting the diffusion of small molecules by the steric effect. This point will be clarified by evaluations using larger permeant molecules. On the other hand, pH and ionic strength appeared to affect the swelling, releasing, and permeation behaviors of films. Despite

this prominent change in the electrostatic state inside films, no significant difference was observed in the permeation coefficient between MB and OR at higher pH. Furthermore, no significant differences were observed in their diffusion coefficients or partition coefficients. These results were understandable based on the absence of a significant effect of swelling on permeation behaviors as discussed above. It is difficult to explain the effects of the charge of permeants on permeation behaviors at higher pH by assuming the partition mechanism. In contrast, MB and OR exhibited similar permeation behaviors at higher pH, which is reasonable by assuming the pore model in which permeants permeate through pores in the membrane by diffusion. These results suggest that CS/CHI films permeated permeants at lower pH using the partition mechanism, and at higher pH, in which films were swollen and the polymer network became larger, films transported permeants using the pore model.

3.3. Effects of the Charge of Permeants on Permeation Behaviors in CS/CHI Films

The effects of the charge of permeants on permeation behaviors in CS/CHI films under low pH conditions in which permeation proceeded based on the partition mechanism were investigated using zwitterionic Trp (Figure 1B) and nonionic MUG (Figure 1B) in addition to monocationic MB and monoanionic OR. Figure 5A shows the permeation behaviors of CS/CHI films in PB at pH 5.9, in which the Trp molecule carries no net electrical charge.
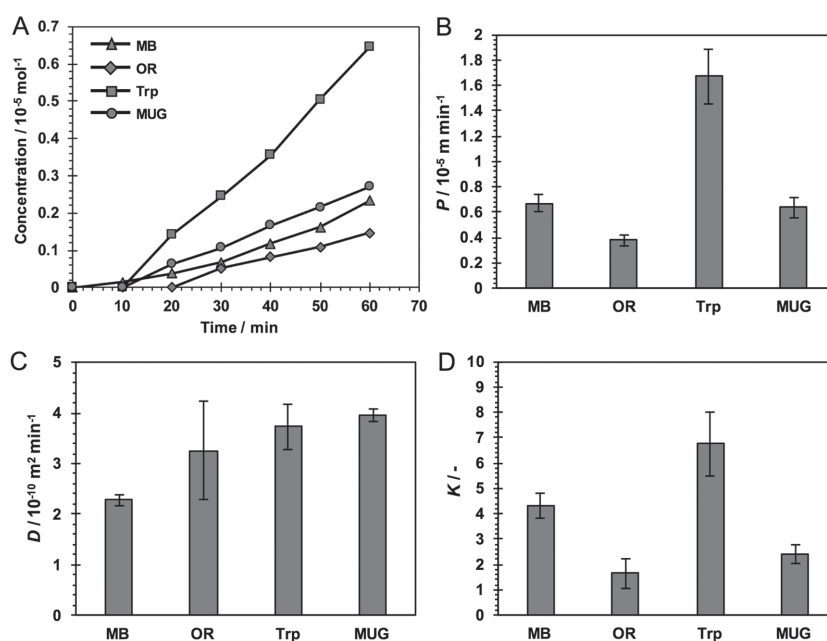

Figure 5. A) Permeation behaviors of CS/CHI films using MB, OR, L-tryptophan (Trp), and 4-methylumbelliferyl β-D-galactopyranoside (MUG) in 10×10^{-3} M PB (pH 5.9), B) permeability coefficients P, C) diffusion coefficients D, and D) partition coefficients K.

As shown in Figure 5B, the order of the permeability coefficient *P* was as follows: Trp > MB ≈ MUG > OR. Zwitterionic Trp carrying zero net charge at pH 5.9 permeated markedly faster than nonionic MUG. The diffusion coefficient *D* and partition coefficient *K* were then calculated. MB showed a lower diffusion coefficient than those of the other permeants (Figure 5C). As discussed above, there was a weak anionic environment inside the films at pH 5.9 (Figure 4B); therefore, diffusion was weaker because of electrostatic interactions between MB and the anionic domain inside the films.

On the other hand, the order of the partition coefficient was as follows: Trp > MB > MUG > OR (Figure 5D). Zwitterionic Trp molecules were the most stable in films due to the double interaction between carboxylic acid groups in Trp and ammonium ion groups in CHI, and ammonium ion groups in Trp and carboxylic acid and sulfate groups in CS. Cationic MB was also more stable than MUG and OR in the anionic environment inside the films than MUG and OR. OR showed a low partition coefficient because of electrical repulsion inside the films. These results support CS/CHI films permeating these molecules by the partition model under low pH conditions.

4. Conclusions

In the present study, the permeation behaviors of CS/CHI films were assessed using four substrates with almost the same sizes, but different charges. The differences observed in the diffusion coefficients and partition coefficients under different conditions indicated that CS/CHI films permeated molecules using the partition mechanism at lower pH, whereas a pore model was dominant at higher pH. CS/CHI films comprised the PICs of CS and CHI and there were still unused ionic functional groups that may interact with the permeants. The permeants were dissolved in and diffused through the films at lower pH, at which the most important factor was the charge of permeants. In contrast, at higher pH, the films swelled more and small molecules such as MB and OR permeated the films by diffusion. In this permeation process, the most important factor was the molecular size of the permeants. These properties may be affected by the molecular size of the permeants; therefore, the measurement of the MWCO of CS/CHI films under various pH represents a forthcoming challenge. The second aim of this study was to evaluate CS/CHI films as separation membranes. The results obtained indicated that CS/CHI films have selective permeabilities based on molecular charges at lower pH and molecular sizes at higher pH, respectively. The utilization of abundant polysaccharides such as CHI as separation membranes is interesting from the viewpoint of the utilization of biomass.

Supporting Information

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