CHAPTER VI
INFLUENCE OF CHARGE CHARACTERISTIC OF SILK FIBROIN ON SORPTION AND RELEASE OF CHARGED DYES

6.1 Abstract

The present study was designed to examine the influence of charge characteristic of silk fibroin on the sorption and release of charged dyes, by varying the pH values of sorption and release media. Negatively charged dyes—Phenol red and Chromotrope 2R—and positively charged dyes—Crystal violet and Indoine blue—were used as the model compounds. Silk fibroin films were prepared by using a solution casting technique. The prepared films were then treated with an aqueous methanol solution or annealed with water to control their conformation. The sorption behaviors of the model compounds by the methanol-treated and water-annealed silk fibroin films were compared. It was found that the fibroin films were amphiphilic materials with the isoelectric point (pI) ranging from 3.8 to 4.5. The methanol-treated films showed a higher sorption of the hydrophobic dyes, as compared to the water-annealed films. The strong interaction was observed when the charges of dye and film were opposite. In contrast, the weak interaction was found in the presence of the similar charges of dye and film. Not only did the charge of the matrix influence the sorption and release behaviors, but the hydrophobicity of the matrix also affected sorption and release. A condition providing the good dye sorption gave the low dye release kinetics.

Keyword: Silk fibroin; Charge; Sorption; Release; Drug delivery

6.2 Introduction

Among various types of biopolymers, silk fibroin, derived from silks of *Bombyx Mori* silkworm, is one of the most promising biomaterials used for biomedical

applications due to a number of reasons. For example, silk fibroin is available in large supply, has been long proven used as medical suture for century. It also possesses excellent mechanical properties. Moreover, it can be solubilized and then further processed into a number of forms through various processing options via aqueous-based or organic-based systems (Vepari and Kaplan, 2007).

Silk fibroin belongs to a group of high molecular weight organic polymers. Its structure consists of repetitive hydrophobic and hydrophilic peptide sequences. Normally, the conformations of the silk fibroin materials can be classified into three forms: random coil, silk I, and β-sheet (silk II) structures. The silk I and β-sheet structures, forming crystalline regions of the fibroin proteins, are dominated by the hydrophobic domains (Mita et al., 1994; Yamada et al., 2001; Asakura et al., 2004). The conformations of the silk fibroin films can be controlled by physical or chemical treatment. For example, the transition of random coil conformation to β-sheet structure was induced by physical stretching, methanol treatment (Jin et al., 2005), laser light treatment (Tsuboi et al., 2001), or heat treatment (Saitoh et al., 2004), while the silk I structure was formed by water annealing (Jin et al., 2005). When compared to physical treatments, the induction of conformation transition by chemical treatment was more widely used because of their simplicity and mild operation conditions. It was reported that the methanol-treated films showed higher hydrophobicity, were stiffer in mechanical properties, and were slower in both enzymatic degradability (Jin et al., 2005) and drug release (Hofmann et al., 2006), as compared to the water-annealed films.

In drug-delivery systems, the use of silk fibroin as the drug-delivery matrix has received considerable attention in recent years (Bayraktar et al., 2005; Hofmann et al., 2006; Cheema et al., 2007; Uebersax et al., 2007; Wang et al., 2007a,b,c). Some of them used the fibroin proteins to control the sorption and/or release of small- and large-charged molecules, or to coat on charged microspheres. Electrostatic interactions were frequently assumed to govern the response of the fibroin proteins to those charged drugs or microspheres. However, it is known that the fibroin proteins contain hydrophobic domains (at least 85%) with a very small portion of charged amino acids. Therefore, it is

interesting to investigate the charge characteristic of silk fibroins in order to fully understand their charged behavior and the role of hydrophobic domains along the fibroin proteins for drug-controlled release. This understanding might be useful and applied for further investigation of silk fibroin as a matrix of charged drug or protein delivery.

In the present contribution, the study was designed to examine the influence of charge characteristic of silk fibroin on sorption and release of charged dyes, by varying the pH values of the sorption and release media. Sorption and release experiments were performed because the sorption and release can imply the affinity and types of interactions between a charged drug and a polymer matrix (Tabata et al., 1998; Serizawa et al., 2005). A transport mechanism of each dye from the polymer films was also determined from a release experiment. In this present study, it was also designed to compare the dye partitioning by methanol-treated or water-annealed silk fibroin films.

6.3 Experimental

6.3.1 Materials

Raw silk fibers, *Bombyx mori*, were obtained from Queen Sirikit Sericulture Center, Saraburi Province (Thailand). Phenol red (dye content 90%, MW 376.36, Sigma-Aldrich), Chromotrope 2R (dye content 75%, MW 468.37, Aldrich), Crystal violet (dye content 90%, MW 407.98, Sigma-Aldrich), and Indoine blue (dye content 70%, MW 506, Aldrich) were used as the model compounds. (The chemical structures of each dye are shown in Figure 6.1) All other chemicals were reagent grade and used as received.

6.3.2 Preparation of silk fibroin films

The preparation of the fibroin solutions was done as previously described (Wongpanit et al., 2007). Briefly, the raw silk fibers of *Bombyx mori* were boiled for 15 min in an aqueous solution of 0.05% Na_2CO_3 and were then rinsed thoroughly with hot water. This process was repeated. After drying at 40°C, the degummed silks were

dissolved in $CaCl_2$:Ethanol:H_2O (molar ratio = 1:2:8) at 78°C and the silk dissolution was completed within 15 min. The solution was filtrated with a filter cloth. The filtrated solution was subsequently dialyzed against distilled water for 4 days (changing the media daily), followed by centrifugation at 10,000 rpm for 10 min. The centrifugation was repeated. The as-prepared aqueous silk fibroin concentration was 6.32 wt%.

To prepare the film, the silk fibroin solution was diluted to 6 wt%, and 200 µl of the diluted solutions were subsequently pipetted into each well of a 48 multi-well plate (COSTAR®, Coming Inc., NY, USA). The solutions were dried overnight at 40°C. The as-cast films were then treated with 90% (v/v) aqueous methanol or were annealed with water. After 30 min, the methanol-treated or water-annealed silk fibroin films were left until dry at 40°C.

6.3.3 Dye sorption

In the sorption test, the experiment was done by using the films which were still on the 48 multi-well plate throughout the study. In order to prevent the evaporation of water during the test, a rubber sheet (kindly supplied by Perfect Built Co., Ltd.) coated with aluminum foil and an additional two layers of flexible polyethylene film was attached to the cover to tightly close the plate.

In order to study the influence of conformations of the silk fibroin on the sorption behavior, the methanol-treated and water-annealed silk fibroin films were compared using Phenol red, Chromotrope 2R, Crystal violet, and Indoine blue as the model compounds. The methanol-treated or water-annealed silk fibroin films were pre-swelled in water for 24 h and then allowed to equilibrate to the aqueous dye solutions (0.6 ml) at an initial concentration of 132 µM. After 24 h, a remaining concentration of the dye solutions after sorption was analyzed using a UV-vis spectrophotometer (Perkin Elmer, Lambda 10). The λ_{max} for Phenol red, Chromotrope 2R, Crystal violet, and Indoine blue were 423, 530, 303 (using 587 nm for a lower concentration range — 0 to 24 µM) and 555 nm, respectively. The results were compared in terms of the partition coefficient, K (see section 6.3.4 for details).

To study the effect of the pH values of the media on the sorption of charged dyes to silk fibroin, Chromotrope 2R and Crystal violet were used because the absorbance of these compound solutions was not sensitive to the studied pH range. The Chromotrope 2R or Crystal violet was dissolved in a buffer solution of 0.04 M acetic/phosphoric/boric acid to achieve a dye concentration at 150 μM (the desired pH of a buffer solution was adjusted by a suitable volume of 0.2 M NaOH solution). After pre-swelling at the same pH value of sorptive conditions for 24 h, the methanol-treated silk fibroin films were allowed to equilibrate with the aqueous dye solutions for 24 h. Thereafter, a remaining concentration of the dye solutions was analyzed using UV-vis spectrophotometry. The results were expressed in terms of the partition coefficient, K.

6.3.4 Partition coefficient calculation

The partition coefficients, K, were calculated as the equilibrium ratio of the concentration of model dye in the film to the dye concentration in the solution using equation 1 (Hunt et al., 1990):

$$K = \frac{C_{film}}{C_{solution}} \qquad (1)$$

$$= \frac{V_s(C_0 - C_{solution})}{V_m C_{solution}} \qquad (2)$$

where C_{film} is the concentration of dye in the film, $C_{solution}$ is the concentration of dye after sorption, C_o is the initial concentration of dye, V_s is the volume of the dye solution, and V_m is the volume of the hydrated film.

The volume of the hydrated film was estimated from its weight and density. The density was determined in a separate experiment in which the diameter, thickness, and weight of a disk of the film were measured after hydration (Hunt et al., 1990).

6.3.5 Density

For determination of the density of the hydrated methanol-treated and water-annealed silk fibroin films, both kinds of films were submerged in water for 1 h

and then punched out to obtain disks of ~6.4 mm in diameter. The disks were further submerged in a pH 7.0 buffer solution at 30°C for 2 days. The densities of the hydrated films were determined using equation 3:

$$D = \frac{W_s}{V_m} \qquad (3)$$

where W_s is the weight of a hydrated film at 30°C and V_m is the volume of a hydrated film calculated from its thickness and diameter (N=15) (Hunt et al., 1990).

To measure W_s, the hydrated films were removed from the test conditions and then gently blotted with a lint-free wipe to remove surface moisture prior to weighing.

6.3.6 Dye release

The release of dyes from the methanol-treated films was studied by adding the release media over the dye-loaded film, still on the well plate, under strong shaking at 30°C. Dye release was assumed to occur from one side of the film only, since the film adhered to the well plate throughout the experiment.

To study the effect of the pH value of the media on the release behavior, the methanol-treated silk fibroin films were loaded with dye at an appropriate pH. Then, the dye-loaded films were used for a release study. Briefly, the methanol-treated silk fibroin films were pre-swelled with a buffer solution at the same pH value as the sorptive conditions for 24 h. Thereafter, the hydrated films were allowed to equilibrate with the dye solutions using Chromotrope 2R (Crystal violet) at 132 μM, pH 2.0 (pH 7.0) with a volume of 1.3 ml. This pH value was chosen because the highest loading was achieved. After 24 h, the remaining dye concentration in the solution was determined to calculate the amount of loaded dye in the films. The dye solution was then replaced by 1.0 ml of the release media of pH 3.0, 4.4, 5.4, or 7.0. The release media was periodically replaced with the 1.0 ml of fresh media and the concentration of the release dyes was analyzed using UV-vis spectrophotometry. Note that the release of dyes at those pH levels was investigated because the fibroin films exhibited different charges at

different pH levels—a positive charge in pH 3.0, a (roughly) neutral charge in pH 4.4, and a negative charge in pH 5.4 and 7.0.

6.3.7 Characterization of methanol-treated and water-treated silk fibroin films

Attenuated total reflectance-fourier-transform infrared (ATR-FTIR) spectroscopy was used to verify the conformation of as-cast, water-annealed, and methanol-treated silk fibroin films. The measurements were carried out on a Thermo Nicolet Nexus 671 FTIR. Each spectrum was acquired in absorbance mode on ZnSe ATR crystal by the accumulation of 256 scans with a resolution of 4 cm^{-1}

6.4 Results and Discussion

6.4.1 Partitioning of charged dyes by methanol-treated and water-annealed silk fibroin films

6.4.1.1 Comparison of the partitioning between the methanol-treated and water-annealed films

It is well known that the conformation of the crystalline structure in the fibroin materials plays an important role, reflecting a number of characteristics, in drug release, biodegradability, mechanical properties, and surface wettability. In the present study, the conformation of the fibroin proteins in the films was controlled by treatment with an appropriate medium to investigate the influence of the conformation of the silk fibroin on the dye partitioning. The structural changes in the fibroin films induced by direct treatment with methanol or by annealing with water were evidenced by ATR-FTIR spectroscopy (see Figure 6.2). The as-cast films exhibited a predominantly amorphous, with some silk I, structure (1638 cm^{-1}, amide I, see Figure 6.2a). After annealing with water, the silk I structure was predominant (1640 cm^{-1}, amide I; 1536 cm^{-1}, amide II, see Figure 6.2b). Upon exposure to aqueous methanol solution, the characteristic absorption shoulder and peak of the β-sheet structure were observed at 1695 cm^{-1} and 1619 cm^{-1}, respectively (see Figure 6.2c) (Jin et al., 2005).

Thus, the conformations of the methanol-treated and water-annealed silk fibroin films were predominantly β-sheet and silk I structure, respectively.

In order to study the partitioning of dyes by the fibroin films, the partition coefficients, *K*, of each dye/film pair were determined. In general, partition coefficients for solutes equilibrated with hydrogel with a *K* value greater than one indicate that a solute prefers to interact with the hydrogel network over the solvent. When the *K* value is less than one, the solute prefers to interact with the solvent over the hydrogel network. When the *K* value is equal to one, the solutes favor neither the hydrogel network nor the solvent (Gehrke et al., 1997). Table 1 shows the partition coefficients, *K*, of the methanol-treated films in comparison with the water-annealed films after sorption of Phenol red, Chromotrope 2R, Crystal violet, or Indoine blue for 24 h.

According to Table 1, the partition coefficients of the sorption of the negatively charged dyes to the methanol-treated films (Phenol red and Chromotrope 2R) are statistically comparable to those of the dye sorption to the water-annealed films, whereas the partition coefficients of the sorption of the positively charged dyes (Crystal violet and Indoine blue) to the methanol-treated films are statistically significantly higher than those of the dye sorption to the water-annealed films.

When comparing the methanol-treated and water-annealed films in terms of their properties, there are two main differences: density and hydrophobicity. In the former, the density of the methanol-treated films; 1.22±0.02 $g \cdot cm^{-3}$ was slightly higher than that of the water-annealed films; 1.18±0.02 $g \cdot cm^{-3}$ (even though they were not significant), so the volumes of the hydrated films of the water-annealed films were slightly higher than those of the methanol-treated films after being equilibrated with water. When dye was absorbed in the water-annealed films, the higher volumed hydrated films led to lower dye concentration in the films. The lower the dye concentration in the films resulted in the lower partition coefficient will be (see Eq. 1).

In regards to the hydrophobicity, Jin *et al.* (2005) reported that the water contact angles of the methanol-treated silk fibroin films were significantly higher

than those of the water-annealed films, implying that the surface of the methanol-treated films was much more hydrophobic. The higher hydrophobicity of the fibroin films was mainly the result of the β-sheet formation during methanol treatment. The β-sheet formation did not occur only at the film surface, but also within the interior of the films (Yoshimizu et al., 1990). During methanol treatment, the water molecules in the water/methanol mixture (90% aqueous methanol solution) penetrated to swell the films. Then, methanol molecules induced the formation of a higher-order conformation of silk fibroin, and most of the hydrophobic groups, such as methyl groups of the hydrophobic domains, were exposed to the hydrophobic-methanol molecules. Thus, the stable β-sheet structure caused the film to be higher in hydrophobicity.

In the case of the water-annealed films, the water molecules induced the silk I formation of the films during water annealing where water-compatible groups would be exposed to water. When compared to the water-annealed films, the methanol-treated films should be more hydrophobic. Thus, the higher hydrophobicity of the methanol-treated films caused greater sorption of the hydrophobic molecules like Crystal violet and Indoine blue. Crystal violet and Indoine blue are classified in the group of hydrophobic compound because they possess a number of methyl groups in their structure. The greater sorption is reflected by a higher K value. It is important to note that the hydrophobicity of the materials has a stronger effect than the density factor because comparable K values were observed in the case of hydrophilic molecules—Phenol red and Chromotrope 2R (see Table 6.1). The hydrophobicity of dyes affecting their sorption to the fibroin films will be discussed in detail in section 6.4.2.

6.4.1.2 Influence of charge on the partition coefficient

As shown in Table 6.1, most of the dyes exhibited a K value greater than one, so all of the model compounds (except Chromotrope 2R), both positive and negative, preferred to interact with the fibroin films over the water. However, the preference to the fibroin films of the positively charged dyes was much greater than that of negatively charged molecules.

To understand the sorption behavior of charged-model compounds by the fibroin films, the chemical structure of the fibroin proteins and their charge characteristic should be considered. Silk fibroin contains at least two major fibroin proteins—light and heavy chains, 25 and 350 kDa, respectively (Altman et al., 2003). When considering the peptide sequence of the heavy chain, the chain can be divided into two regions: smaller, internal, hydrophilic blocks and internal hydrophobic blocks. These two blocks alternatively connected to each other for twelve domains with larger hydrophilic blocks at the chain ends (Foo et al., 2006). For the amino-acid sequences of hydrophobic regions (e.g., a simple description of the hydrophobic sequence is GAGAGS; where G, A, and S are Glycine, Alanine and Serine, respectively), these sequences dominated the β-sheet and/or silk I structure forming the crystalline part of the fibroin material and contributed no charge to the protein chains (Mita et al., 1994). In addition, the sum of the amino-acid fraction of Glycine (43-45%), Alanine (30%), and serine (12%) is around 85-87% (McGrath, 1998; Khan et al., 2007). Thus, the fibroin proteins possessed mostly hydrophobic-uncharged fractions.

In contrast, the amino-acid sequence of the hydrophilic part possessed a variety of charged amino acids. For example, the positively charged amino acids are Arginine and Lysine, while the negatively-charged amino acids are Aspartic acid and Glutamic acid (Foo et al., 2006). Therefore, these proteins are the amphiphilic compounds. At neutral pH (the condition of this experiment), the fibroin proteins exhibited a net negative charge because the isoelectric point (pI) of these proteins is in the range of 3.8-4.5 (see Table 6.2). As a result, the positively charged dyes favored much more to adsorb to the protein chains than the negatively charged dyes because of the attraction forces between opposite charge of positively charged dyes and the fibroin films.

However, Phenol red showed a partition coefficient greater than one, meaning that the dye, which is the negatively charged compound, should have interaction with the fibroin matrices as well. As mentioned above, repulsion could occur between the negative charge of the fibroin chains and the dye molecules. However, most

of the fibroin chains exhibited no charge, and the polar groups along the fibroin chains (such as amide linkages) were able to interact with dye molecules when they had a contact to each other. Accordingly, the partition coefficient of the negatively charged Phenol red was higher than one. Interestingly, the *K* value of Chromotrope 2R was very close to one. This could be caused by the presence of two negatively charged groups in a single dye molecule. Thus, the strong repulsion of two negatively charged groups to the negative charge of the fibroin proteins compensated the other interactions of the dye with the fibroin chains.

6.4.2 Influence of pH value on sorption and release behavior

6.4.2.1 Sorption

In section 6.4.1.2, the influence of the charge on the dye sorption to the fibroin films has already been studied, and the results suggested that the opposite charges caused strong interaction between the dye molecules and the fibroin chains. In contrast, the weak interaction (low values of partition coefficients) was observed in the presence of the similar charges of dye molecules and silk fibroin. However, the effect of pH on the dye sorption to the fibroin films should be further studied in order to confirm this suggestion because type of the charge and charge intensity along the fibroin chain could be affected by the pH of the sorption media.

Figure 6.3 shows the influence of pH value on the sorption of Chromotrope 2R and Crystal violet. Apparently, the partition coefficients of Chromotrope 2R were dramatically decreased from 1946 to only 32.7 when the pH increased from 2.0 to 4.0. However, beyond pH 4.0, the partition coefficients were gradually decreased and reached 2.19 eventually at neutral. In contrast, the partition coefficients of Crystal violet were substantially increased from 182 to 3295 when the pH of the buffer solution increased from 2.8 to 7.0.

As shown in Table 6.2, the pI of the fibroin proteins is in the range of 3.8-4.5. When these proteins were in a buffer solution at a pH value lower than

pI, the total charge of the proteins was positive. In contrast, if the pH value is greater than the pI of silk fibroin, the total charge is negative.

Considering a pH value of the buffer solution lower than the pI of silk fibroin, the negatively charged dye (Chromotrope 2R) could interact with the positive charge of the proteins via electrostatic interaction, as the partition coefficient could be observed to be largest at the lowest pH (K = 1946). However, the electrostatic interaction was weaker at higher pH because the intensity of the positive charge along the protein chains was lower. This led to the partition coefficient of Chromotrope 2R being lower. On the other hand, in the case of Crystal violet, this dye displayed a similar charge as the fibroin proteins in this considered pH range (lower than pI). Thus, the repulsion could occur, but the partition coefficient of the Crystal violet showed a relatively large number even at pH 2.8 (K = 182; this will be discussed later). When pH further increased, the partition coefficients of Crystal violet were much higher because the lower intensity of the negative charge along the protein chains caused a lower repulsion force between the protein chains and dye molecules.

When the pH of a buffer solution was higher than the pI of silk fibroin, the fibroin proteins became negatively charged. Thus, repulsion between the negatively charged dye (Chromotrope 2R) and the proteins occurred. This force was more intensive when the pH of the media became higher because the intensity of the negative charge along the fibroin chains increased as the pH of the media rose. Accordingly, the partition coefficients of Chromotrope 2R gradually decreased when the pH rose. In contrast, the positively charged dye—Crystal violet—favored interaction with the fibroin proteins due to the electrostatic interaction, so the partition coefficient increased when the pH of the media increased.

Interestingly, although Crystal violet exhibited a similar charge of the fibroin proteins at a pH lower than the pI of silk fibroin, the partition coefficients of Crystal violet were relatively large (such as at pH 2.8, the K value was 182). It is known that the fibroin structure is composed of hydrophobic and hydrophilic domains in the same chains. The domain that strongly responds to changeable charge character is the

hydrophilic domain, which is a small portion along the protein chains (less than 13-15%). Furthermore, not all hydrophilic domains contain charged (Foo et al., 2006). Thus, besides the charge of the proteins affecting the sorption of the dye molecules, the hydrophobicity of the matrix was an important factor as well in determining the ability of the dye molecules to be adsorbed to the protein chains. In this case, the hydrophobicity of the matrix played a very important role because the partition coefficients of Crystal violet were large even though the matrix possessed the same charge.

When the fibroin matrix existed in the media at a pH above pI—displaying a negative charge opposite to the charge of Crystal violet—the K values were much greater than those of the matrix in the media at a pH below pI (see Figure 6.3). Thus, the partition coefficients of Crystal violet could be the result of the synergism of hydrophobicity and charge of the fibroin proteins when pH of media was above pI. To simplify the above description, the schematic illustration of the pH-dependent interactions between dyes and the fibroin matrices after dye sorption is showed in Figure 6.4. As shown in this Figure, the negatively charged dye prefer to interact with hydrophilic domains of the fibroin chains via electrostatic attraction and polar-polar interaction, while the positively charged dye prefer to interact with negatively-charged hydrophilic domain by electrostatic attraction as well as with hydrophobic domains via hydrophobic interaction.

6.4.2.2 Release

Typically, to avoid the possible cumbersome exact analysis of the data, an empirical equation can be used to find a mechanism of drug release. Ritger and Peppas (1987) proposed a simple empirical equation to describe the release mechanism of solute release from swellable matrices, including sheets (films), cylinders, spheres, and polydisphere samples (valid for $M_t/M_\infty < 0.6$):

$$\frac{M_t}{M_\infty} = kt^n \qquad (4)$$

where M_t/M_∞ is the fraction of drug release, k is the diffusion constant, t is the release time, and n is the diffusional exponent. Fickian diffusion and Case-II transport from a sheet are defined by n = 0.5 and n = 1.0, respectively, whereas anomalous (non-fickian) transport is defined by $0.5< n <1.0$.

Usually, the swellable polyelectrolyte matrices of both anionic and cationic types provide an initial $t^{1/2}$ dependence (n = 0.5) (Ottenbrite and Kim, 2001). In the present study, the mechanisms of dyes released from the silk fibroin films of both negative and positive charge were found to be fickian diffusion as well. Charged and uncharged compounds released from the poly(vinyl alcohol) films also obeyed with this empirical equation (Taepaiboon et al., 2006). Thus, this empirical equation proposed by Ritger and Peppas was able to be used in various types of released compounds—both charged and uncharged molecules.

Figure 6.5 shows the release profiles of Chromotrope 2R and Crystal violet at different pH levels of buffer solutions from the silk fibroin films. By plotting between the fraction of dye release against square root of time, the initial slope of the line shows a good linear relationship with acceptable r^2 (see Table 3). As can be observed from the diffusion constants of dyes in each pH value in Table 3, the increase of the pH of buffer solutions for the Chromotrope 2R resulted in an increase of the release kinetics, while the opposite results were obtained in the case of the Crystal violet.

This was caused by an increase in the intensity of the negatively-charged pendant groups along the fibroin chains as the pH of the buffer solution increased. Thus, the charge of the fibroin proteins changed from positive charge at pH 3.0 (below pI) to around neutral at pH 4.4 (around pI), to negative at pH 5.4, and more in negative charge at pH 7.0 (above pI) as the pH increased. For a negatively charged dye, the interaction was changed from electrostatic attraction at pH 3.0 to electrostatic repulsion at pH 7.0. As a result, the release kinetics increased as the pH of the buffer solution increased. On the other hand, the opposite results were observed in the case of Crystal violet because the attraction forces between the positively charged dye and the

fibroin chains were stronger as the pH of the buffer solution increased. Thus, the higher attraction force between the dye and the matrix as the pH increased caused the lower dye release kinetics. Additionally, the incomplete release of the positively charged dye was observed. As can be seen in Figure 6.5B, the highest release fraction was only 0.55 at release media pH 3.0 even though the fibroin matrix and the Crystal violet possessed the same charge. This is mainly caused by the strong hydrophobic interaction between the fibroin proteins and dye molecules.

At this point, considering the correlation between diffusion constants and partition coefficients (see Table 6.3), it can be concluded that the higher partition coefficient gave the lower diffusion constant. In the other word, lower release kinetics was observed when the interaction between dye molecules and the polymer chains was stronger.

The results of this study suggest that not only did the charge of the fibroin chains influence the sorption and release of the charged dyes, but their hydrophobicity also affected sorption and release. This suggestion may be very important for understanding in further study in drug-controlled release particularly in protein delivery. For localized protein delivery, proteins can immobilize to a carrier matrix by covalent bonding, physical entrapment, adsorption or ionic complexation (Luginbuehl et al. 2004). Among them, the immobilized proteins in a carrier matrix via ionic complexation can be successfully applied for many of *in vivo* studies with maintaining their biological activity (Young et al., 2005; Tabata, 2005). However, Luginbuehl et al. (2004) suggest that to design appropriate protein/carrier system with maintaining protein bio-activity, in addition to electrostatic interaction, the hydrophobic interaction may be taken into account. Now, we are studying the interaction and controlled release of basic fibroblast growth factor (bFGF, pI 9.6) using silk fibroin as a carrier matrix. This will be the subject of a future publication.

6.5 Conclusion

It is known that many characteristics of the fibroin films were affected by the method of film treatment. In the present study, the methanol-treated and water-annealed films also showed different sorption behavior. The higher hydrophobicity of the methanol-treated films compared to the water-annealed films caused greater sorption of the hydrophobic dye. By varying the pH values of sorption and release media as well as using a various types of model compounds, the charge characteristic of silk fibroin shows pH-dependent properties. Not only did the charge of the fibroin chains influence the sorption and release of the charged dyes, but their hydrophobicity also affected sorption and release. This is caused by the structure of the silk fibroin, which is a block co-polymer comprising hydrophilic and hydrophobic domains. Most of the amino acids of the fibroin chains were hydrophobic fractions (around 85-87%). By using the empirical equation, the analyzed mechanism of transport of the charged dyes, both negative and positive, from the fibroin films was fickian diffusion. The results from the present study suggest the general behavior of silk fibroin for basic essential knowledge for further utility in drug-delivery systems, particularly in the controlled-release design strategy involving complexation—electrostatic interactions with a charged drug.

6.6 Acknowledgements

The authors would like to acknowledge Professor Pitt Supaphol for the stimulating discussion. This work was supported by the Thailand Research Fund (through the Royal Golden Jubilee Ph.D. Program, i.e. RGJ grant) and The Petroleum and Petrochemical College, Chulalongkorn University. The authors wish to thank Queen Sirikit Sericulture Center (Saraburi Province, Thailand) and Perfect Built Co., Ltd. (Thailand) for supplying essential materials for this study.

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Table 6.1 Comparison of the values of partition coefficients between methanol-treated and water-annealed silk fibroin films of each dye.

Type[b]	Partition coefficient, K[a]			
	Phenol red	Chromotrope 2R	Crystal violet[c]	Indoine blue[c]
Methanol-treated films	4.02±0.14	1.00±0.21	3015±10	733±2.7
Water-annealed films	3.63±0.24	0.955±0.17	2701±6.4	538±4.2

[a] = Data were expressed as the mean ± the standard deviation of the data.

[b] = Densities of the methanol-treated and water-treated silk fibroin films were 1.22±0.02 and 1.18±0.02, respectively.

[c] = Partition coefficients of the methanol-treated films were statistically significantly higher than those of water-annealed films with $P<0.05$ (the unpaired Student's t-test).

Table 6.2 Isoelectric points of the fibroin proteins determined from various methodologies.

Isoelectric point	Method of determination	Method of Regeneration		Reference
		Degumming	Dissolving	
3.9	Electrophoretic mobility	0.05% Na_2CO_3	$CaCl_2$/Ethanol/Water	Malay et al., 2007
4.22	Calculation	-[b]	-[b]	Zhou et al., 2000
3.8-3.9	-[a]	0.5% Na_2CO_3	$CaCl_2$/Ethanol/Water	Ayub et al., 1993
4.5	Membrane potential	0.5% Na_2CO_3	$CaCl_2$/Ethanol/Water	Chen et al., 1994
4.5	Membrane potential	0.5% Na_2CO_3	$CaCl_2$/Ethanol/Water	Cheng et al., 2005

[a] = The authors raised without any comment.

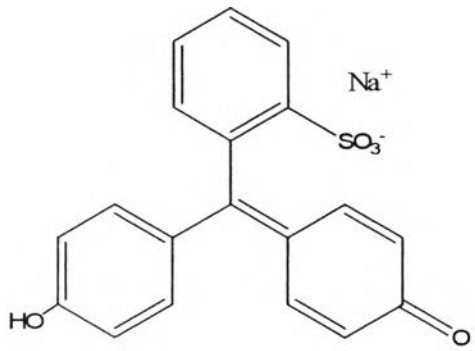
[b] = The raw sequence data were obtained by the Fragment Assembly program in Seqlab of the Genetics Computer Group sequence analysis software package version 10.0.

Table 6.3 Diffusion constants of Chromotrope 2R and Crystal violet at various pH values of the release media obtained by calculation of the initial slope of each curve in Figure 6.5C &D in comparison to their partition coefficients at the same pH.

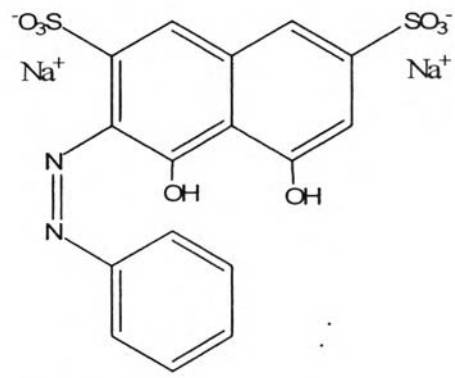
pH	Chromotrope 2R (-)			Crystal violet (+)		
	$k \times 10^3$ ($min^{-1/2}$)	r^2	K	$k \times 10^3$ ($min^{-1/2}$)	r^2	K
3.0	9.17[a]	0.99	908[b]	11.1[a]	0.99	257[b]
4.4	39.7	0.97	30.4	4.15[a]	0.99	1508
5.4	47.5	0.96	8.70	0.669[a]	0.99	2158[b]
7.0	49.0	0.96	2.10	0.352[a]	0.97	3295

[a] = Analysis based on the first seven release points of the data of Figure 6.5C & D (beyond this, the curvature was observed).

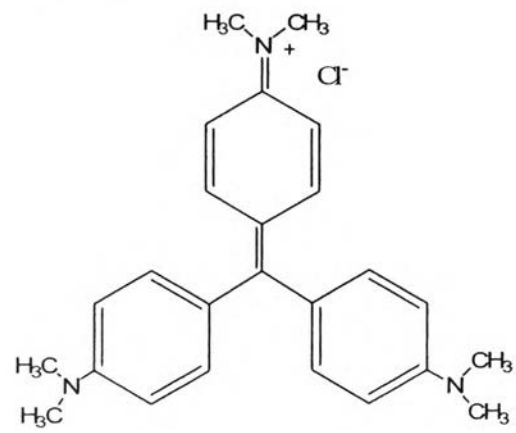
[b] = Partition coefficient was obtained from the data in Figure 6.3 by a mathematical estimation.



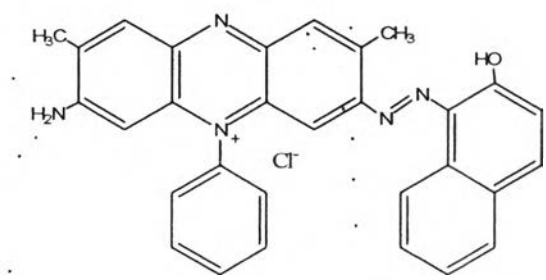
(A) MW 376.36



(B) MW 468.37



(C) MW 407.98



(D) MW 506

Figure 6.1 Chemical structures of (A) Phenol Red, (B) Chromotrope 2R, (C), Crystal violet and (D) Indoine blue.

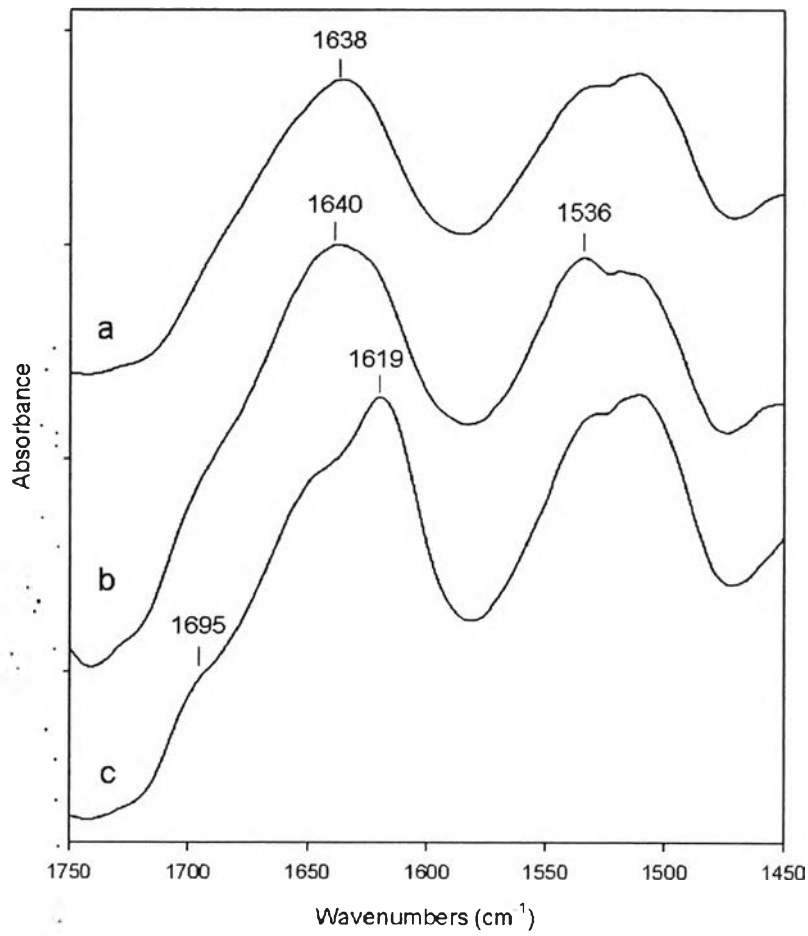


Figure 6.2 ATR-FTIR spectra of a) as-cast, b) water-annealed and c) methanol-treated silk fibroin films.

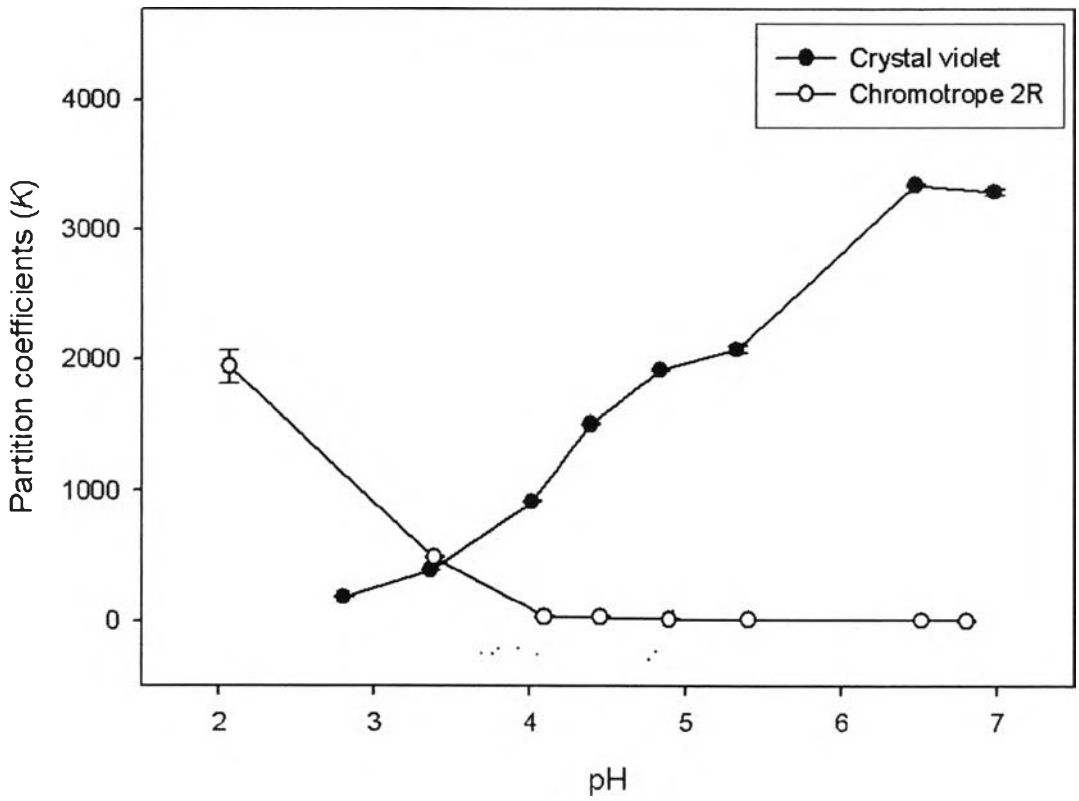


Figure 6.3 Partition coefficients of (o) Chromotrope 2R and (•) Crystal violet by silk fibroin films at 30°C as a function of pH.

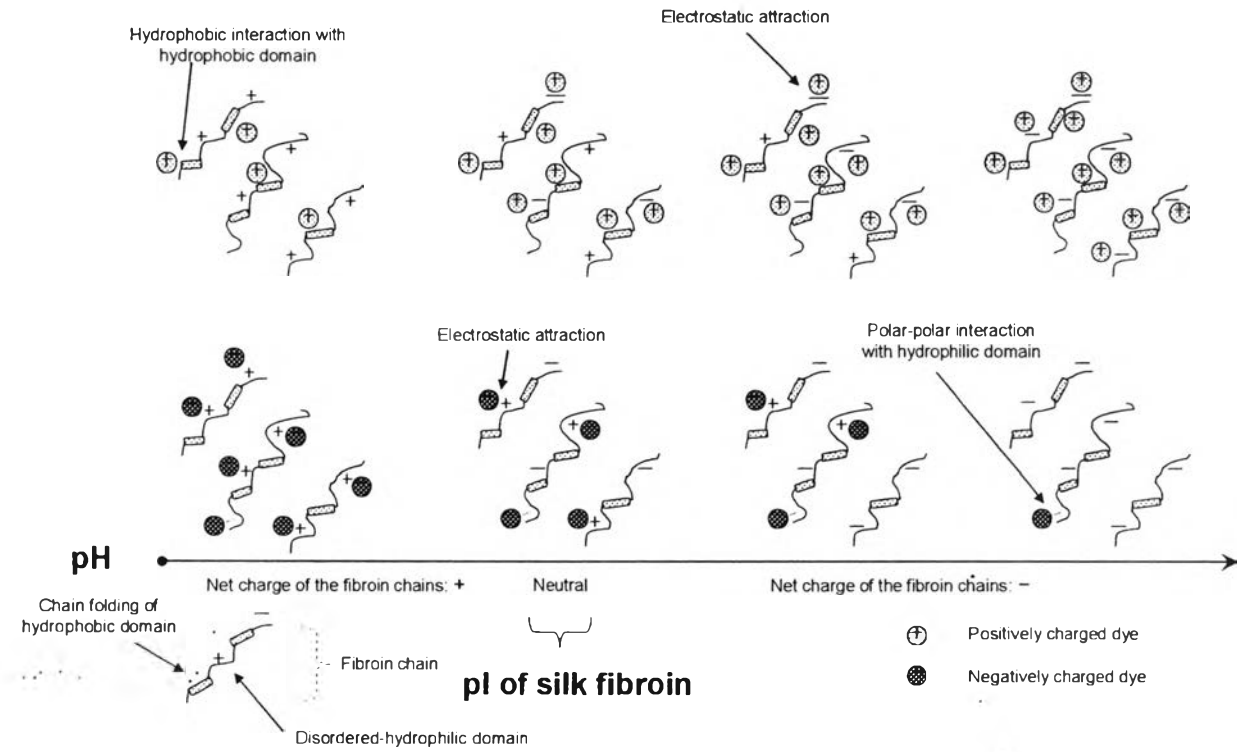


Figure 6.4 Schematic illustration of the interactions between dyes and fibroin chains as a function of pH.

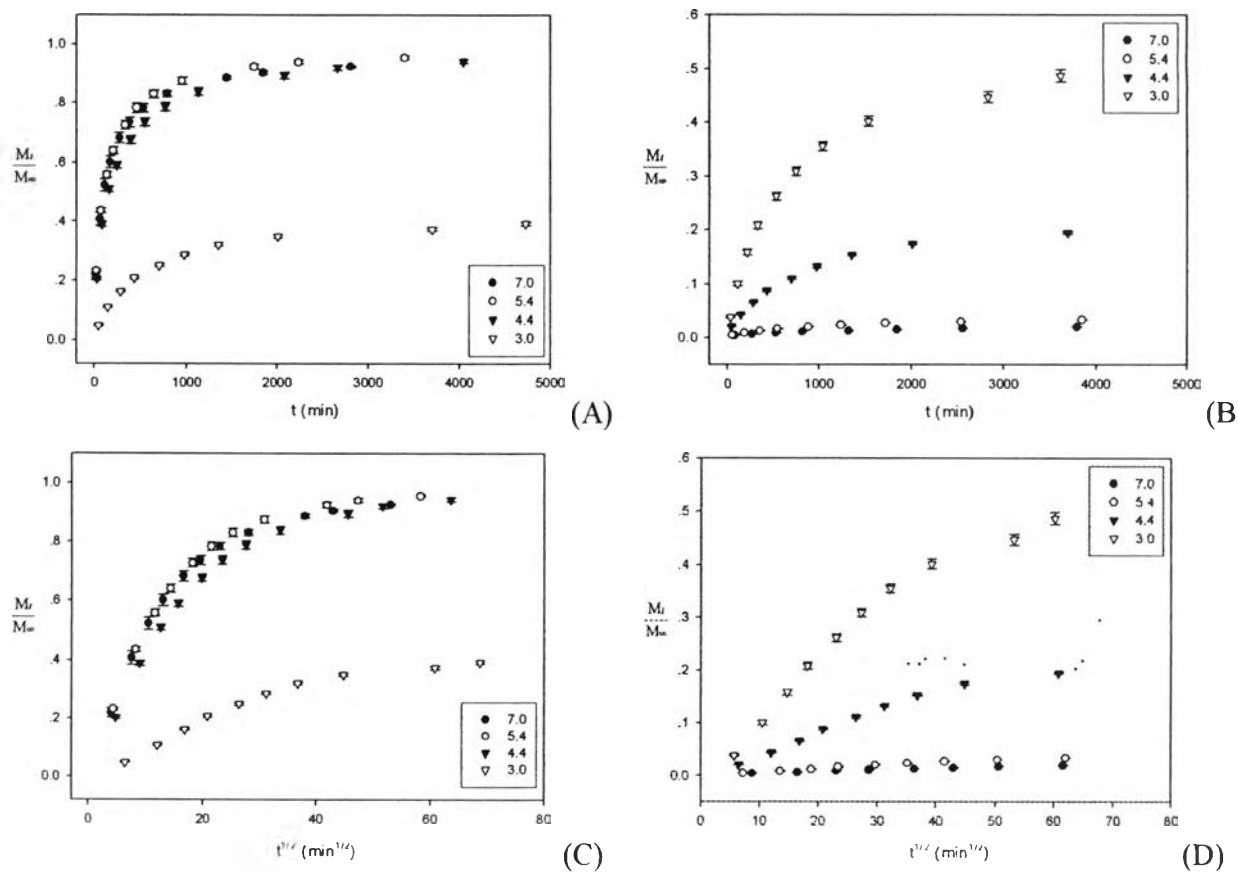


Figure 6.5 Release profiles of Chromotrope 2R (A & C) and Crystal violet (B & D) from dye-loaded silk fibroin films in different pH media.