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Diffusion characteristics of collagen film

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Abstract

Collagen films prepared by treating collagen gel solutions with different concentrations of glutaraldehyde were evaluated as a biodegradable and biocompatible drug carrier for cosmetically effective agents in this study. The influences of concentration of glutaraldehyde (0, 0.05, 0.075, 0.1, 0.2, 0.25, and 0.3%, v/w) with a fixed concentration (1%, w/w) of collagen on the crosslinking rate of collagen gel solutions and on the crosslinking extent of the collagen contained within were examined by monitoring changes in viscosity. In addition, the influences of the addition of different model drugs (retinoic acid, retinol palmitate, ascorbic acid 6-palmitate, and tocopherol acetate) on viscosity changes of collagen gel solutions were compared. The results demonstrate that the maximal viscosity of collagen gel solutions increases with increasing concentrations of glutaraldehyde. When the concentration of glutaraldehyde exceeds 0.2%, the maximal viscosity of collagen gel solutions reaches a plateau. However, model drugs showed insignificant effects on viscosity changes of collagen gel solutions. The diffusion characteristics of collagen films prepared from those gel solutions crosslinked with different concentrations of glutaraldehyde were assessed using two different matrix forms of solution or gel for the model drugs in a flow-through diffusion system. The matrix effect on the flux of model drugs from both solution and gel matrix through collagen films was inconclusive. However, both fluxes show the same tendency to decrease when the concentration of glutaraldehyde used for crosslinking is increased. However, when the concentration of glutaraldehyde exceeds 0.2%, these model drugs, except retinoic acid, show similar diffusion characteristics across the collagen films.

Keywords: Collagen film; Glutaraldehyde; Diffusion; Viscosity; Vitamins

1. Introduction

Natural polymers are increasingly being studied for controlled-release applications because of their biocompatibility and biodegradability. Various materials such as hyaluronic acid [1,2], fibrinogen [3], fibrin [4,5], and collagen [6,7] have been tested as carriers for drug delivery systems. Collagen is a potentially useful biomaterial since it is a major constituent of connective tissue. Collagen is unique in possessing different levels of structural order: primary, secondary, tertiary, and quaternary. In vivo, type I collagen molecules are stacked together in orderly arrays called fibrils. Fibrils are strengthened by two types of covalent crosslinks: intramolecular and intermolecular. Covalent intermolecular crosslinks between collagen molecules in macromolecular fibrils are essential for stability and are respon-

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sible for various physicochemical properties. Biomaterial made of collagen offers several advantages: it is biocompatible and non-toxic to most tissues; it has well-documented structural, physical, chemical, and immunological properties; it can be processed into a variety of forms; and it is readily isolated and purified in large quantities.

Several factors can affect both the structural integrity of collagen films and the diffusion rate of drugs through collagen in this form. In a previous publication [7], we discussed the characteristics of collagen isolation and application of collagen gel as a drug carrier. However, the rate of drug release from collagen matrices can be modified by treating the collagen matrices with a suitable crosslinking agent. Glutaraldehyde is the common choice for a crosslinking agent. It is a mild bi-functional agent, which forms a bridge between fibers by reacting with the ε-amino groups of lysine residues in proteins [8]. The mechanism of its interaction with proteins is complex and not clearly defined. Whatever the mechanism, the ensuing crosslinking is able to modify the integrity or pore size and, in turn, affects the rate of drug diffusion through collagen films.

Glutaraldehyde is now the most widely used reagent for crosslinking. It has previously been used as a crosslinker to produce albumin microbeads with various gel structures [9]. However, glutaraldehyde-crosslinked biomaterials can induce local cytotoxicity [10]. Therefore, it is important to minimize the amount of glutaraldehyde used. In this study, the effect of different concentration of glutaraldehyde used in preparing collagen films on the diffusion characteristics of model drugs (retinoic acid, retinol palmitate, ascorbic acid 6-palmitate, and tocopherol acetate) in two different matrix forms of solution or gel was examined. Optimally, a minimal concentration of glutaraldehyde should be elucidated for controlling the diffusion of model drugs through collagen films. The purpose of selecting these model drugs was to examine the possibility of using collagen films as a carrier to topically deliver these cosmetically effective agents.

2. Experimental methods

2.1. Materials

Porcine skin was collected from a local slaughterhouse or seller. Pepsin (pepsin A; 91 units/mg), all-*trans*-retinol acetate, ascorbic acid 6-palmitate, (±)-α-tocopherol acetate, and a 25% glutaraldehyde aqueous solution were obtained from Sigma (St. Louis, MO, USA). Acetic acid, phosphoric acid, propylene glycol, sodium azide, retinol palmitate, and ascorbic acid were supplied by Merck (Germany). Retinoic acid was obtained from Hoffmann-LaRoche (USA). Cremophor RH 40 (polyoxyl 40 hydrogenated castor oil) was provided by BASF (Germany).

2.2. Methods

2.2.1. Collagen

Telopeptide-poor collagen was isolated from porcine skin using a procedure reported previously [7]. Generally, fresh porcine skin was obtained, and the hair and fat were removed by scraping and acetone treatment. The treated skin was then homogenized and digested with pepsin (Sigma) at pH 2.0 (in HCl with the addition of 0.1% NaN_3) for a desired period of time (~20 h). The pH of the supernatant was adjusted to 10.0 with NaOH, after which it was allowed to stand for 24 h to inactivate the pepsin; then it was readjusted to pH 7.0 with HCl. The precipitated collagen was then dissolved in a 3% acetic acid solution and freeze-dried.

2.2.2. Measurement of viscosity of collagen gel solutions

The viscosity of collagen gel solutions was measured using a Brookfield Viscometer (model LVDVII+CP). First, a collagen solution at 1% (w/w) was prepared by dissolving collagen in 3% acetic acid. After complete dissolution, model drugs (retinoic acid at 0.01%, and retinol palmitate, ascorbic acid 6-palmitate, and tocopherol acetate at 0.1%) were added as designated. Then the gel solutions were put into a jacketed container to measure the viscosity. A circulating water bath was connected to this jacketed container for maintaining the temperature of the container at 25°C. Before adding various concentrations of glutaraldehyde (0, 0.05, 0.075, 0.1, 0.2, 0.25, and 0.3%, v/w), a suitable spindle was mounted and activated with a desired rotating speed, and measurements were taken after the designated

temperature was reached and a stable reading was recorded. The viscosity was recorded at predetermined time intervals. An average of three replicates was reported for each time point.

2.2.3. Collagen films

A collagen solution at 1% (w/w) was prepared by dissolving collagen in 3% acetic acid. Then various concentrations of glutaraldehyde (0, 0.05, 0.075, 0.1, 0.2, 0.25, and 0.3%, v/w) were added, and these mixtures were immediately transferred into a round acrylic mold, which had been sealed with parafilm paper (American Can, USA) around the bottom. These molds were then placed in an electric dryer (Intech, Osaka, Japan) until the weight of the collagen film approached a constant value. Then, the parafilm paper was carefully peeled off, and the collagen films were stored in a desiccator at ambient temperature until use. The thickness of the collagen films was measured at five different randomly selected places. The uniformity of the thickness of collagen films was acceptable since the coefficient of variation was less than 5%.

2.2.4. Diffusion studies

Diffusion studies were carried out using retinoic acid (0.01%), retinol palmitate (0.1%), ascorbic acid 6-palmitate (0.1%), and tocopherol acetate (0.1%) as model drugs either in solution or a gel form (using 1%, w/w, collagen as a gelling agent and 10% Cremophor RH40 as a solubilizer) at 37°C, in a flow-through diffusion system. This system consists of a multi-channel peristaltic pump (202U/AA, Watson Marlow), a fraction collector (Retriever IV, ISCO, USA), a circulating water bath, and six units of flow-through diffusion cells. The flow-through diffusion cells contain two side arms, which enable the conduction of receiver-cell media from a peristaltic pump to a fraction collector. The temperature was maintained at 37°C by circulating constant-temperature water through the outer jacket of the receiver cell. The surface area of the receiver cell opening was 1.77 cm^2, and the volumes for the donor and receiver compartments were 0.8 and 0.1 ml, respectively. A 0.9% normal saline solution (containing 0.01% sodium azide and 10% Cremophor RH40) was used as the receiver cell medium. The receiver cell media were stirred at 450 rpm by externally driven, teflon-coated magnetic bars. Collagen films prepared with different concentrations of glutaraldehyde were mounted onto each receiver cell, and an O-ring and cell top were placed on the top of each membrane. These components were then clamped securely in place. The receiver cell medium reservoir was maintained at 37°C. Subsequently, a solution or gel form of the model drugs was applied on the top of each collagen film. All samples were collected over 10-h periods. The diffusion of model drugs through collagen films was followed by determining their concentrations in the collected medium as a function of time. The concentrations of these model drugs were analyzed by a validated HPLC method as described below.

2.2.5. Analytical procedures

The HPLC system consisted of a pump (Jasco, model PU-980), a wavelength changeable UV-detector (Jasco, model UV-975), an automatic injector (Jasco, model 851-AS), a reversed-phase cartridge column (C8, 5 μm, Lichrospher® 250×4 mm, Merck), and a computer integrator. The flow rate was 1.0 ml min^{-1}. In the case of retinoic acid and ascorbic acid 6-palmitate, the mobile phase was composed of methanol and 10 mM phosphoric acid in a volume ratio of 90:10. UV detection was at the wavelengths of 340 and 245 nm for retinoic acid and ascorbic acid 6-palmitate, respectively. The retention times of retinoic acid and ascorbic acid 6-palmitate were around 9.5 and 4.2 min, respectively. For retinol palmitate and tocopherol acetate, the mobile phase was composed of methanol and 10 mM phosphoric acid in a volume ratio of 95:5. UV detection was at the wavelengths of 325 and 284 nm for retinol palmitate and tocopherol acetate, respectively. The retention time of tocopherol acetate was 12.4 min, whereas it was 24.3 min for retinol palmitate. The HPLC method was validated with an acceptable coefficient of variation for accuracy and precision for all model drugs.

2.2.6. Partition coefficient measurements

The partition coefficient for retinoic acid into the collagen films was determined. Pieces of round collagen films (volume: 0.1097 ± 0.005 cm^3) were presoaked in saline solution. The film was wiped dry and transferred to 3.0 ml of a retinoic acid-con-

taining solution (1.7973 μg/ml). The collagen films were allowed to equilibrate at 37°C for 30 h (a period that has been validated to establish equilibrium). Equilibrium concentrations of retinoic acid were determined using the HPLC method described above. Partition coefficients (K) were calculated based on the following equation:

$$K = (C_0/C_{eq} - 1) \cdot V/V_f$$

where C_0 is the initial concentration of solute, C_{eq} denotes the concentration of solute in the buffer after equilibrium, and V and V_f are the volumes of buffer and collagen film, respectively. An average of three replicates was reported.

2.3. Data analysis

The flow-through diffusion cell system can be assumed to undergo instantaneous mixing in the receiver cell. The mass balance equation that governs the concentration change in the receiver cell is given by

$$\frac{dC(t)}{dt} = \frac{AJ(t)}{V} - \frac{F_0 C(t)}{V} \quad (1)$$

where $C(t)$ is the concentration of solutes in the receiver cell, A is the diffusion area, $J(t)$ is the input rate from the donor cell, V is the volume of the receiver cell, and F_0 is a constant flow rate from the peristaltic pump to the receiver cell. The related initial condition is that $C(t)$ is equal to 0 at $t = 0$. Under steady-state conditions, $J(t) = J_{ss}$, Eq. (1) can be solved for $C(t)$ as

$$C(t) = \frac{A \times J_{ss}}{F_0}\left(1 - \exp\left(-\frac{F_0 t}{V}\right)\right) \quad (2)$$

Eq. (2) describes the concentration profile of solutes in the receiver cell as a function of time. The concentration in the receiver cell ($C(t)$) increases with time and approaches the plateau value of $(A \times J_{ss})/F_0$ as time approaches infinity [11]. Then, Eq. (2) is integrated to obtain the cumulative amount of solutes collected ($Q(t)$) for steady state as the following equation:

$$Q(t) = A \times J_{ss}\left(t - \frac{V}{F_0}\right) \quad (3)$$

The value of J_{ss} was calculated from the slope of the linear plot of the cumulative amount per unit area ($Q(t)/A$) of solutes versus time, which is equivalent to that obtained by Eq. (4). According to Fick's first law, the steady state flux (J_{ss}) is constant and is expressed by

$$J_{ss} = \frac{DK}{h}(C_d - C_r) \quad (4)$$

where D and K are the diffusion and partition coefficients, and C_d and C_r are the concentrations of solutes in the donor cell and receptor cell, respectively. For retinoic acid, a saturated solution (C_d = solubility) was maintained in the donor compartment, and sink conditions were maintained in the receptor compartment. Using the partition coefficients determined above, diffusion coefficients were then calculated with back substitution according to Eq. (4) with h being designated as the thickness of the collagen films.

3. Results and discussion

The natural polymer of teleopeptide-poor collagen has low antigenicity and is biocompatible, biodegradable, and less toxic than synthetic polymers. Glutaraldehyde crosslinking of collagen significantly reduces the antigenicity and biodegradation [12]. The influence of glutaraldehyde on physical properties of type I collagen molecules after crosslinking to different extents was studied by measuring the viscosity change of collagen gel solutions, and the results are shown in Fig. 1 (0% glutaraldehyde as the control). The viscosity of collagen gel solutions gradually reached a plateau and became constant after a sufficient time of crosslinking reaction for all levels of glutaraldehyde concentrations. On the other hand, the viscosity at the plateau also increased with increasing concentration of glutaraldehyde and became constant when the concentration of glutaraldehyde used exceeded 0.2% at a 1% (w/w) concentration of collagen. This indicates a gradual transformation of the liquid characteristics of the collagen solution into solid characteristics as the concentration of glutaraldehyde used increases.

The crosslinking rate was estimated from the slope of the initial portion of the curve, and the viscosity at

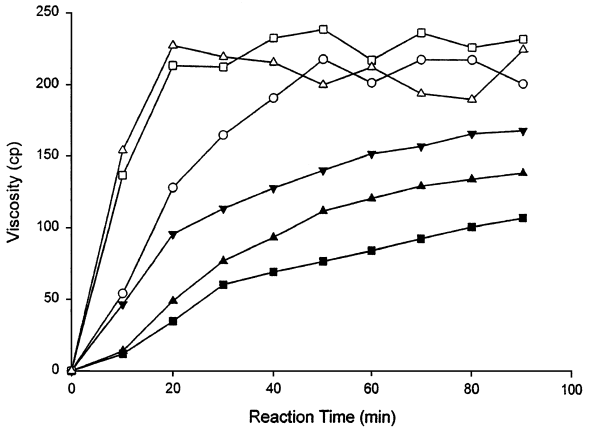

Fig. 1. Effect of different concentrations of glutaraldehyde on viscosity changes of collagen gel solutions: (■) 0.05%; (▲) 0.075%; (▼) 0.1%; (○) 0.2%; (□) 0.25%; (△) 0.3%.

the plateau was designated as the maximal viscosity of the collagen gel solution, which is correlated with the extent of crosslinking. Results in Table 1 show that both the crosslinking rate of collagen gel solutions and the maximal viscosity increased with increasing concentrations of glutaraldehyde and then reached a plateau when the concentration of glutaraldehyde exceeded 0.2%. With increasing concentration of glutaraldehyde, the crosslinking rate between glutaraldehyde and lysine residues provided by the fixed amount of collagen should increase as a result of increasing collision frequency between glutaraldehyde molecules and the terminal amino moiety of lysine. On the other hand, the maximal viscosity reflects the extent of crosslinking. For a fixed amount of lysine residues on collagen available for crosslinking, the extent of crosslinking should gradually approach completeness with an increasing concentration of glutaraldehyde, with the maximal viscosity in turn gradually reaching a plateau. These results appear to be in agreement with some data from the literature [5]; this suggests that glutaraldehyde concentrations are important in determining the nature of the crosslinked network generated.

Table 1
Crosslinking rate and maximal viscosity for collagen gel solutions treated with different concentrations of glutaraldehyde

Glutaraldehyde (%)	Crosslinking rate (cp/min)	Maximal viscosity (cp)
0.05	2.03	107.54
0.075	2.36	138.89
0.1	3.89	168.58
0.2	4.92	211.23
0.25	10.66	230.53
0.3	11.35	210.51

The effects of model drugs (retinoic acid, retinol palmitate, ascorbic acid 6-palmitate, and tocopherol acetate) on viscosity changes of collagen gel solutions were also examined. It was observed that there were no significant alternations of viscosity of collagen solutions in the presence of each individual model drug. A minimal interaction between collagen fibrils and these model drugs was expected due to the hydrophobic nature of these model drugs with a limited amount of each being solubilized in the collagen solution. As a result, the influence of the presence of these model drugs on the viscosity change of collagen gel solutions was observed to be insignificant.

Experiments were conducted to examine the diffusion characteristics of collagen films without crosslinking or with crosslinking with different concentrations of glutaraldehyde prepared from collagen gel solutions following the procedure described in Section 2. Model drugs, including retinoic acid, retinol palmitate, ascorbic acid 6-palmitate, and tocopherol acetate, were prepared in two different matrix forms of either solution or gel. The values of steady state flux (J_{ss}) of model drugs through the collagen films were obtained from the slopes of the linear portion of the plots in Fig. 2 based on Eq. (4); results are summarized in Table 2. Plots of the values of steady state flux (J_{ss}) of the solution and gel forms of the model drugs versus glutaraldehyde concentration are also shown in Fig. 3. Values of J_{ss} decrease with increasing concentration of glutaraldehyde for retinol palmitate, ascorbic acid 6-palmitate, and tocopherol acetate in both solution and gel matrices. Furthermore, these values of J_{ss} approach a constant when the concentration of glutaraldehyde used for crosslinking exceeds 0.2%. However, the matrix effects on the flux of model drugs from both solution and gel matrix through collagen films were inconclusive.

The extent of crosslinking of collagen films prepared by treatment with different concentrations of glutaraldehyde is listed in Table 1. Values of J_{ss} decrease correspondingly with an increasing extent

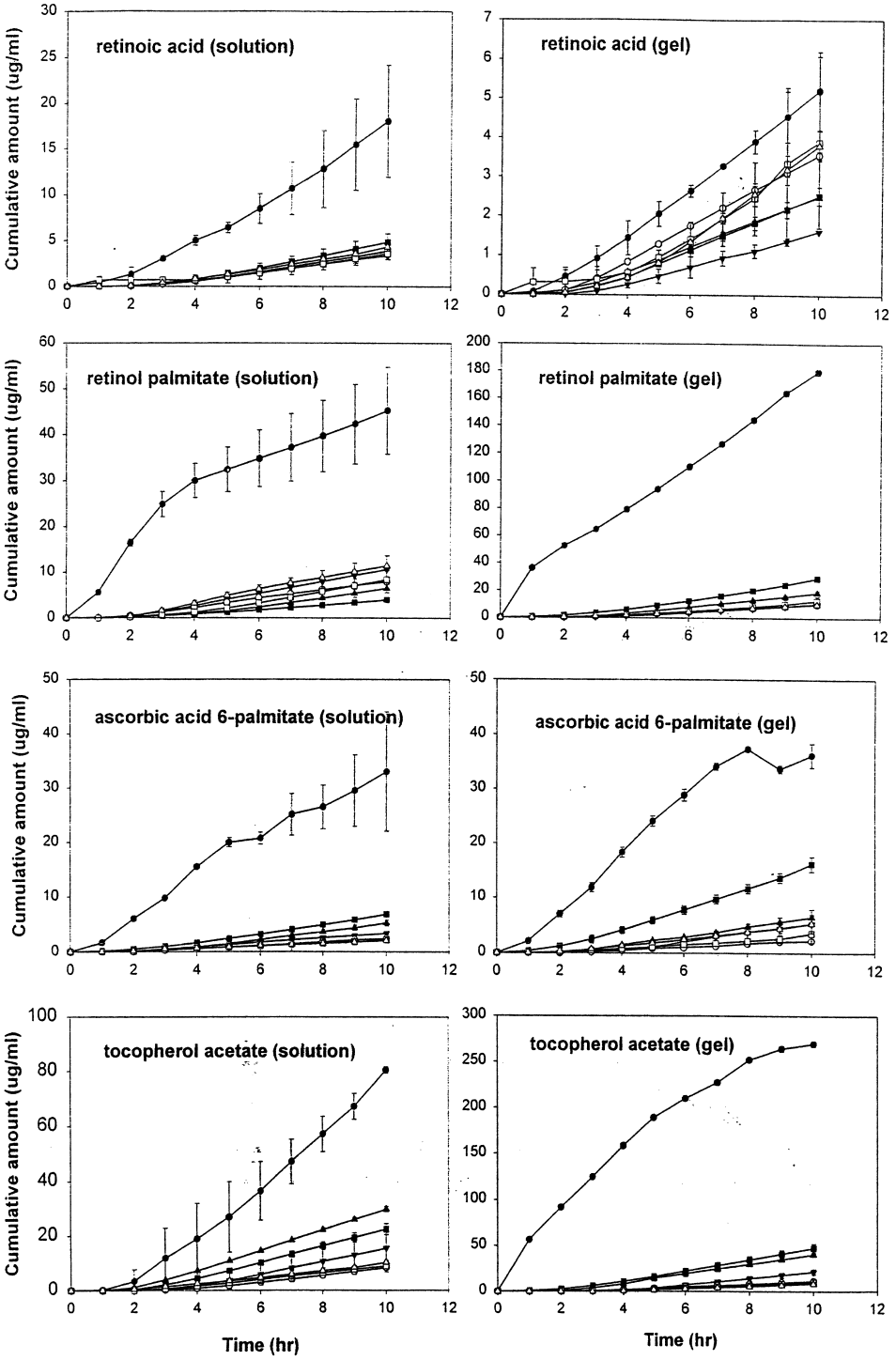

Fig. 2. The cumulative amount of model drugs penetrating versus time with either solution or gel form through collagen films treated with different concentrations of glutaraldehyde: (●) 0%; (■) 0.05%; (▲) 0.075%; (▼) 0.1%; (○) 0.2%; (□) 0.25%; (△) 0.3%.

Table 2
Values of J_{ss} ($\mu g\ cm^{-2}\ h^{-1}$) for model drugs across collagen films crosslinked with different concentrations of glutaraldehyde and the extent of crosslinking of these collagen films

Glutaraldehyde (%)	Degree (%) of crosslinking	Ascorbic acid 6-palmitate		Tocopherol acetate		Retinol palmitate		Retinoic acid	
		Solution	Gel	Solution	Gel	Solution	Gel	Solution	Gel
0	0	16.89±8.16[a]	19.76±2.43	50.20±9.87	63.19±6.42	12.58±1.93	37.16±8.09	12.09±4.72	3.27±0.86
0.05	66.55±1.11	4.84±0.52	9.69±0.04	30.21±1.55	30.20±4.71	5.44±1.71	20.83±4.02	3.43±0.51	1.79±0.06
0.075	80.01±1.59	3.63±0.29	6.84±3.09	18.56±0.74	28.34±6.47	5.09±0.03	13.01±5.28	2.91±1.74	1.57±0.004
0.1	90.15±1.27	1.98±0.04	4.20±2.24	11.82±1.96	17.28±1.13	5.47±0.97	8.33±0.51	2.31±0.13	1.19±0.03
0.2	>100	1.73±0.39	1.11±0.45	6.60±2.09	7.42±0.12	4.45±0.72	6.79±1.12	1.94±0.26	1.62±0.18
0.25	>100	1.63±0.69	1.98±2.16	5.89±0.80	11.97±6.25	4.88±1.30	7.40±2.13	6.50±0.47	3.08±1.27
0.3	>100	0.99±0.23	2.40±2.40	5.77±0.68	6.58±1.14	3.24±0.37	6.58±3.45	5.33±0.63	3.46±0.29

[a] Mean±standard error.

of crosslinking, and then gradually approach a plateau when reaching a full extent of crosslinking. The correlation of the change in J_{ss} values with the extent of crosslinking is obvious. Since the maximal viscosity is related to the extent of crosslinking as discussed above, the similarity between the maximal viscosity of collagen solutions in the presence of different concentrations of glutaraldehyde and the diffusion characteristics of model drugs through collagen films treated with glutaraldehyde is expected.

Nevertheless, a significant increase in the values of J_{ss} for retinoic acid through the collagen film was noticed when the concentration of glutaraldehyde used for crosslinking exceeds 0.2%. Since permeability is defined as the ratio of product of the

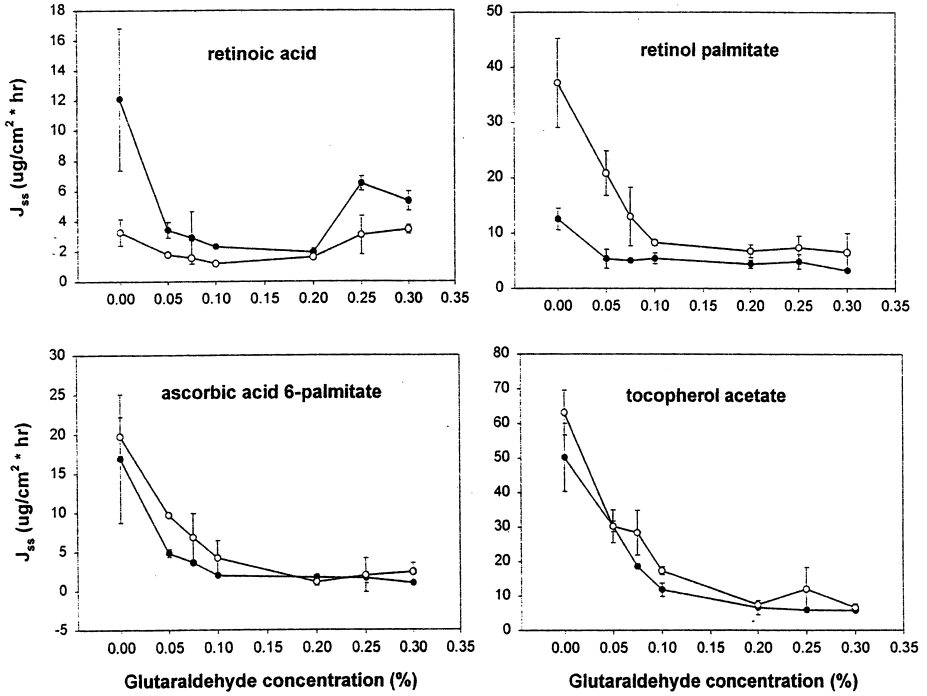

Fig. 3. Plots of values of steady state flux (J_{ss}) of solution (●) and collagen gel (○) form of model drugs versus glutaraldehyde concentration through collagen films.

partition coefficient and the diffusion coefficient to the thickness of the film, it would be more appropriate to examine the separate effects of partitioning and diffusion behavior. Permeability was calculated by dividing J_{ss} by the solubility of retinoic acid in the donor compartment. Diffusion coefficients were determined according to the known partition coefficients and the thickness of collagen films. Variabilities of permeability and in the partition and diffusion coefficients for retinoic acid through collagen films treated with different concentration of glutaraldehyde are shown in Fig. 4. There is a significant reduction in partition coefficients for collagen film crosslinked with various concentrations of glutaraldehyde compared to collagen film with no crosslinking. A tendency for a decreasing partition coefficient with an increasing extent of crosslinking was further observed. However, the extent of the reduction was not as large as that between treated and untreated collagen films. Also, a decrease in the diffusion coefficient with an increasing extent of crosslinking of collagen films was demonstrated initially, but then an increase occurred for those collagen films crosslinked with concentrations of glutaraldehyde exceeding 0.2%.

Actually, the introduction of hydrophobic characteristics into collagen films by crosslinking with glutaraldehyde is expected since two aldehyde groups of glutaraldehyde are connected by a hydrophobic alkyl chain. Therefore, the increase in partition coefficients of collagen films for a hydrophobic compound with no interaction is expected with the increasing extent of crosslinking by introducing more glutaraldehyde. The hydrophilicity of collagen fibrils is further reduced, with the result that most of the hydrophilic ϵ-amine groups of lysine residuals are crosslinked with a hydrophobic crosslinker of glutaraldehyde. The decreasing percentage of free ε-amine groups with increasing concentration of glutaraldehyde for crosslinking has been demonstrated (shown in Table 2) [13]. This result conflicts with what has been observed, i.e., a decrease in the partition coefficient of retinoic acid into collagen films with an increasing extent of crosslinking. A better explanation for this might be that an interaction exists between the carboxylic group of retinoic acid and the ε-amine group of lysine residuals. Retinoic acid will less favorably partition into collagen films when the free ε-amine groups in collagen films become exhausted. Therefore, the increasing hydrophobicity and a less-favorable interaction with the increasing extent of crosslinking create comparable partition coefficients of retinoic acid for col-

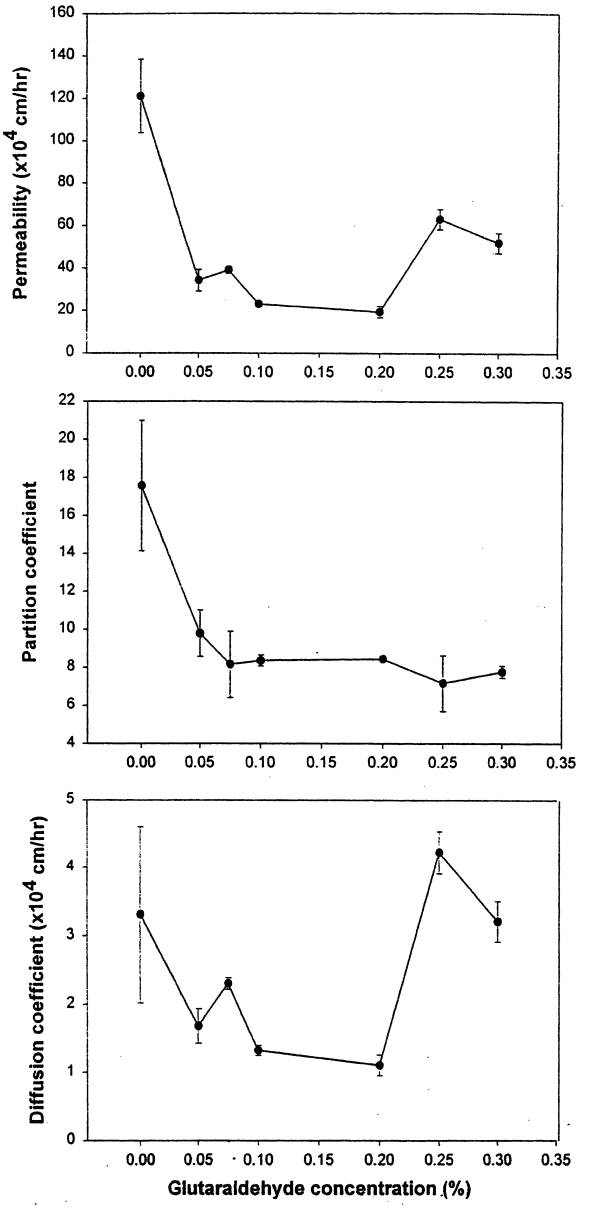

Fig. 4. Variabilities of permeability and partition and diffusion coefficients for retinoic acid through collagen films treated with different concentrations of glutaraldehyde.

lagen films crosslinked with different concentrations of glutaraldehyde as those in Fig. 4.

A more rigid structure and greater entanglement of fibrils are expected in collagen films treated with glutaraldehyde compared to untreated ones, resulting in decreased permeability and diffusion coefficients. Nevertheless, the permeability and diffusion coefficients of retinoic acid decreased correspondingly with the increasing extent of crosslinking, and then increased as the extent of crosslinking exceeded 90%. These phenomena might be a result of the crosslinking of terminal amino groups on collagen approaching completion as the concentration of glutaraldehyde exceeds 0.2%. Therefore, the number of free amine groups on collagen gradually decreased. It has been reported that the diffusion of solute within the film is retarded not only by molecular weight effects, but also by some additional interactions with the film [5]. The extent of interaction during the diffusion process between crosslinked collagen film and retinoic acid seems to decrease with an increasing concentration of glutaraldehyde. As a result, retinoic acid diffuses easily through collagen films crosslinked with higher concentrations of glutaraldehyde.

4. Conclusions

The unique properties of collagen films make them a viable system for drug delivery. As indicated by the transport behavior of model drugs (retinoic acid, retinol palmitate, ascorbic acid 6-palmitate, and tocopherol acetate), collagen films are a suitable carrier. Crosslinking with glutaraldehyde further modifies the diffusion characteristics of collagen films and can be used to manipulate the desired release rate for model drugs. Crosslinking collagen films with glutaraldehyde to the full extent at a concentration of 0.2% seems to be optimal in controlling the diffusion of model drugs and reducing the cytotoxic effect of glutaraldehyde.

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