



※膠原蛋白與自凝型 Hydroxyapatite 膠泥之複合材質的 ※  
※開發與應用※

✓NSC89-2314-B038-~~601~~<sup>810</sup> —

執行期間： 86 年 8 月 1 至 89 年 7 月 31 日

共同主持人：

☐ 國際合作研究計畫國外研究報告書一份

中華民國 89 年 10 月 1 日

# 行政院國家科學委員會專題研究計畫成果報告

## 膠原蛋白與自凝型 Hydroxyapatite 膠泥之複合材質的開發與應用

計畫編號：NSC87-2314-B-038-012

NSC88-2314-B-038-001

NSC89-2314-B-038-001

執行期限：86 年 8 月 1 日至 89 年 7 月 31 日

主持人：許明照 台北醫學大學藥學研究所

### 一、中文摘要

本計畫利用台灣自有的豬皮為材料來源，萃取純化得到不具抗原性的膠原蛋白。並以電泳分析、掃描式電子顯微鏡和分子篩液相層析法等標定所得之膠原蛋白的物理特徵。純化出的膠原蛋白以不同比例的酒精、水及丙二醇混合製造膠原蛋白膜，並以不同碳鏈長度的 paraben 進行穿透試驗。結果顯示，隨著碳鏈長度的增加其穿透力隨之下降。進一步，我們在膠原蛋白膜處方中加入 hydroxypatite，由 x-ray 晶格繞射及電子顯微鏡的拍攝，得知 hydroxypatite 確能在膠原蛋白膠體中合成。且其結構不會因在膠原蛋白膠體中而有所改變，然而膠原蛋白膜的張力會因有 hydroxypatite 的存在而大幅降低。當膠原蛋白膜處方中加入 hydroxyapatite 時，會使 paraben 的穿透力比在不含 hydroxyapatite 更低。相同地膠原蛋白膜中含有 hydroxyapatite 時會使蛋白質藥物的穿透力降低。

膠原蛋白載體的特性可進一步利用交聯劑 (cross-linking agent) 加以修飾，以調控適用於不同藥物特質。由實驗結果顯示膠原蛋白黏度會隨著戊二醛濃度之增加而提高，但當戊二醛濃度達到 0.2% 以上時，其黏度會到達平衡而成一定值。另一方面，添加 Cremopher RH40 時，膠原蛋白黏度反而會隨著 Cremopher RH40 濃度之增加

而降低，但活性藥物卻並不會影響膠原蛋白的黏度。在滲透實驗中，發現大部分藥物溶液或膠體在不同戊二醛濃度交聯的膠原蛋白膜中，滲透通量 (Flux) 皆會隨著戊二醛濃度增加而降低，當戊二醛濃度達到 0.2% 以上時，藥物滲透通量會達到平衡；可是在維生素 A 酸藥物中不論是以溶液或膠體的藥物形式，戊二醛濃度達到 0.2% 以上時，藥物滲透通量反而會有增加的趨勢。

關鍵詞：膠原蛋白，Hydroxyapatite，複合材質，膜衣，藥物載體

### Abstract

In this study, an efficient and simply way to isolate type I collagen from the pig skin was developed and validated. The effects of various purification conditions on the characteristics of telopeptide-poor collagen fiber were compared. It was first confirmed that a 1:50 ratio of pepsin to skin was favorable for the digestion of porcine skin. The morphological characteristics observed by scanning electron microscopy (SEM) showed that fibril collagen, porous fibril membrane or dense membrane were all possibly formed depending on the digestion and freeze-drying media. Analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and size-

exclusion HPLC revealed that the collagen sample produced by digestion with pepsin in a pH 2.5 HCl solution to obtain telopeptide-poor collagen, and freeze-drying in 0.5 M acetic acid for the final step, resulted in less oligomers. Not only were its fibril characteristics qualitatively identical to those of standard collagen but also were easily hydrated suggesting that this procedure is an appropriate choice for both isolation and purification.

Collagen so obtained was processed into film in the mixture containing various ratios of water, propylene glycol, and ethanol. An experimental mixture design was applied to characterize the effect of individual solvent and their interaction on the properties of such a collagen film. Scanning Electronic Microscopy (SEM) was used to examine the surface property of collagen film. Penetration studies using a series of parabens with different alkyl chain length and macromolecules with different molecular weight were used to characterize their physical properties. The tensile strength and related characters of collagen films was evaluated as well using Dynamic Mechanical Analyzer (DMA). The effect of adding Hydroxyapatite (HA) at a concentration of 10 mM on the physical characters of collagen films was evaluated by the same studies as above.

First of all, it was confirmed by SDS electrophoresis and size exclusion HPLC that the process conditions proposed in this study was a simple and reproducible way with a high yield to isolate type I collagen from the pig skin.

By the examination of X-ray and SEM, it was confirmed that hydroxyapatite was formed inside the collagen film. However, the tensile strength of collagen films without hydroxyapatite was 4-5 time higher than that with hydroxyapatite. This is probably due to the discontinuity of film structure caused by the existence of hydroxyapatite in the collagen films. This results in the easiness of breakage

when the force was applied onto the collagen films. The results of statistical analysis of experimental design revealed that whether the existence of hydroxyapatite or not, water added during the preparation of collagen films was the most significant factor that enhancing the tensile strength of collagen films produced.

The permeability of proteins penetrated through the collagen films without hydroxyapatite decreased with the increase of molecular weight of protein and with the added amount of propylene glycol in the film formation. There showed a lower flux for the same protein molecule penetrated through the collagen films with hydroxyapatite than that without hydroxyapatite.

Collagen films prepared by treating with different concentration of glutaraldehyde were evaluated as a biodegradable and biocompatible drug carrier in this study. The influences of adding different concentration of glutaraldehyde (0%, 0.05%, 0.075%, 0.1%, 0.2%, 0.25% and 0.3%) into a collagen gel solution with a fixed concentration (1%) of collagen on the crosslinking rate of collagen gel solutions and on the crosslinking degree of collagen within were examined by monitoring the change of viscosity. In addition, the influence of the addition of different concentration of Cremophor RH40 (1%, 5%, and 10%) or different model drugs (retinoic acid, retinol palmitate, ascorbic acid 6-palmitate, and tocopherol acetate) on the viscosity change of collagen gel solutions were compared. The results demonstrated that the viscosity of collagen gel solutions increased with increasing the concentration of glutaraldehyde. When the concentration of glutaraldehyde exceeded 0.2%, the viscosity of collagen gel solutions reached plateau. Nevertheless, the decreasing extent of viscosity of collagen gel solutions increased with increasing the added concentration of Cremophor RH40. However, model drugs showed insignificant effect on the viscosity of collagen gel solution. The diffusion characteristics of collagen films prepared

from those gel solutions crosslinked with different concentration of glutaraldehyde were assessed using solution form or collagen gel form of model drugs in a flow-through diffusion system. The flux of model drugs from both solution and collagen gel matrix through collagen films decreased when the concentration of glutaraldehyde used for crosslinking increased. But, when the concentration of glutaraldehyde exceeded 0.2%, those model drugs except retinoic acid showed similar diffusion characteristics across the collagen films.

## Introduction

Collagen is a potentially useful biomaterial since it is a major constituent of connective tissue. Its biomaterial characteristics offer several advantages: it is biocompatible and non-toxic in 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. Collagen by itself can be used for short-term implant applications, as it is resorbed within 6-8 weeks. Therefore, many researchers are trying to develop it as various prosthetic and surgical devices including wound dressings and vitreous implants [1]. In addition, collagen has served as an excellent carrier for drug delivery systems. The possible use of collagen film as a vehicle for pilocarpine delivery has been examined by Vasantha et al. [2]. Collagen-poly(HEMA) hydrogels containing 5-fluorouracil for use in chemotherapy for a solid tumor fibrosarcoma in rats showed an improved antitumour activity [3]. More recently, several investigators found that a corneal collagen shield accelerates corneal epithelial healing in experimentally wounded animals. Milani et al. reported that collagen shield delivery of gentamicin-dexamethasone may be comparable to subconjunctival injections providing an alternative therapy after intraocular surgery [4].

Collagen is unique in possessing different levels of structure order: primary, secondary, tertiary, and quaternary. Nearly 15 types of collagen have been reported. Type I collagen has a helical structure which is composed of three polypeptide chains; two identical chains, termed  $\alpha 1(I)$  and one chain, termed  $\alpha 2(I)$ . Each of the 3 chains consists of 1000 amino acid residues with an approximate molecular weight of 100,000. 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. The covalent intermolecular crosslinks between collagen molecules in macromolecular fibrils are essential for stability and various physicochemical properties. After solubilization of collagen from skin, some of the crosslinks are still present. Thus soluble type I collagen preparations generally contain a mixture of monomers, dimers and higher oligomers.

In general, the telopeptide of type I collagen is thought to be responsible for causing an immunogenic response when introduced into xenogenic hosts. To eliminate this problem, solubilized skin collagen can be digested with pepsin to remove telopeptides [5]. The telopeptide-poor collagen is a mixture of monomeric and oligomeric molecules [4]. However, there have been many different isolation and purification procedures described for the preparation of lyophilized telopeptide-poor collagen [6-10]. The procedure described by Ramesh et. al. involved suspending tissue (200 g wet weight) in 1.5L of 0.5 M acetic acid mixed with 100 mg pepsin and incubation at 1°C for 48 h with stirring [7]. Vasantha et al [2] used 0.001 M hydrochloric acid for their preparation of telopeptide-poor collagen by treatment with pepsin (approximate ratio of enzyme to collagen was 1:400) at 20°C with intermittent stirring for 5 days. The main difficulty with all these techniques, which involve various different conditions, is that they can not ensure the quality of the collagen isolated from pig skin.

Collagen has been developed as an attractive biopolymeric carrier for a variety of drugs and can be easily processed in a variety of forms for specific therapeutic purposes. In order to improve the mechanical properties of collagen, cross-linking as well as grafting have been investigated [11-13]. Hydroxyapatite (HAP) is a major component of hard tissue and incorporation of porous HAP with bovine collagen protein has shown to improve the mechanical properties of the composites [14]. However, the combination of these two materials was still by physical mixing. In this study, we compared various isolation and purification procedures for collagen from tissue. Besides that, we tried to grow the HAP directly in the collagen film. The morphology as well as mechanical properties of the preparations were also examined.

Several factors can affect the structural integrity of the collagen films, and the diffusion rate of drugs through collagen in this form. In a previous publication [15] 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 treatment of the collagen matrices with a suitable crosslinking agent. As a crosslinking agent, glutaraldehyde is common and of the choice. It is a mild bifunctional agent, which forming a bridge between fibers by reacting with the  $\epsilon$ -amino groups of lysine residues in proteins [16]. 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, affect the rate of drug diffusion through the collagen films.

Glutaraldehyde is now the most widely used reagent for crosslinking. It has previously been used as a crosslinker to produce albumin microbead with various gel structure [17]. However, glutaraldehyde crosslinked

biomaterials induce local cytotoxicity [18]. Therefore, it is important to minimize the amount of glutaraldehyde used. In this study, the effect on the diffusion characteristics of model drugs (retinoic acid, retinol palmitate, ascorbic acid 6-palmitate and tocopherol acetate) in solution or collagen gel form across collagen films prepared with different concentration of glutaraldehyde was examined. Optimally, a minimal concentration of glutaraldehyde could be elucidated to control the permeation of model drugs through collagen film.

## RESULTS AND DISCUSSION

The natural polymer of telopeptide-poor collagen is of low antigenicity, biocompatible, biodegradable, and less toxic than synthetic polymers. It consists of a mixture of monomeric and oligomeric molecules when extracted from pig skin by pepsin digestion. The oligomers are held together by covalent crosslinks between molecules. There are several factors affecting the characteristics of telopeptide-poor collagens including the ratio of pepsin to skin, temperature, and digestion medium or lyophilization medium. A schematic representation of the telopeptide-poor collagen samples obtained by the different fabrication techniques is illustrated in Fig. 1. Figure 2 shows the percent viscosity change of collagen homogenates digested by various ratio of pepsin to skin (1:50, 1:100 and 1:200) in HCl solution. With increasing time, the homogenate becomes more and more viscous and gelifies even further. The viscosity of the homogenate gradually reaches a plateau indicating complete digestion. The time required for collagen to be completely digested by the ratio of 1:50 was about 16 hours, which was shorter than the time required for collagen to be digested using the ratios of 1:100 or 1:200. Variations in the former process seem to be smaller. The results suggest that the 1:50 ratio of pepsin to skin is workable in terms of the viscosity of the final mixture and the time period needed. Because collagen readily becomes converted

to gelatin (i.e., denatured), special precautions must be taken so that tissue and derived extracts be maintained at appropriate temperature. Thus, tissues must not be permitted to become unduly warm during the grinding or blending procedures; and solutions used for extraction, or washing must be prechilled. A shorter digestion time would be least harmful to the collagen.

The procedures employed for the isolation and purification of telopeptide-poor collagen are outlined in Fig. 1. The designations A/A, A/N, H/H, H/A or H/N were assigned to five different products prepared by different processes. Observation of the appearance and bulk structure of these telopeptide-poor collagens was carried out by scanning electron microscopy (SEM). (Fig. 3). The telopeptide-poor collagen designated A/A is soft in its native form and absorbs water easily and is also easily dispersed. Under SEM (Fig. 3a), the fibrous texture is observed to have a fibril form. The collagen sample designated A/N appears to be a plane-sheet-like film with a dense structure when examined using the naked-eye. Solubilization of this sample in acidic solution was with some difficulty. SEM examination (Fig. 3b) showed a dense film structure with a coarse surface but no fibrillar appearance. Fig. 3c shows a fibrous film with a porous structure and some cracks for the collagen sample designated H/H. This sample was not easily hydrated in acidic solution, and a longer time was needed for it to completely disperse. The collagen sample designated H/A had a similar appearance to that of A/A but was softer. However, it was more easily hydrated and dissolved in acidic solution. A bundle-like structure of fibrils was observed under SEM (Fig. 3d). Compared to A/A, the bundle-like of fibrous structure of H/A was more obvious. A soft porous film which was able to disperse and then dissolve in acidic solution was observed for the collagen sample designated H/N. Its fibrous texture with some porous structures is illustrated in Fig. 3e. From this, the collagen sample designated H/A is the most acceptable based

on the rate and easiness of dissolving in acidic solution.

It has been shown that the fibril assembly of Type I collagen affects the morphology of the collagen fibril [19]. The assembly of aggregated structures of collagen from fully solubilized monomeric molecules, protein-treated molecules, partially solubilized fibrous suspensions, or insoluble swollen, cross-linked fiber networks may be mediated by hydrogen bonding, hydrophobic effects, electrostatic interaction, and entropic and dispersions-forces [20]. The rate of collagen fibril assembly is markedly dependent on temperature and ionic strength, pH and protein concentration. Therefore, different morphologies observed under SEM for collagen samples obtained under various conditions is expected. Although a unified quantitative formalism incorporating all the above factors to describe Type I collagen fibril assembly in vitro has been proposed, it has only been used as a simple, semi-empirical description of the many aspects of collagen fibril assembly.

Fig. 4 shows the SDS-PAGE electrophoresis patterns of the standard and collagen samples. All these collagen sample displayed two  $\alpha$  bands ( $\alpha 1$  and  $\alpha 2$ ,  $M_w=95,000$ ), two  $\beta$  bands ( $\beta 11$  and  $\beta 12$ ,  $M_w=190,000$ ) and two  $\gamma$  bands ( $\gamma 11$  and  $\gamma 12$ ,  $M_w=285,000$ ). One dimensional densitograms of SDS-PAGE electrophoresis for these collagen samples are shown in Fig. 5 and illustrate that the percentage distribution of the  $\alpha 1$  band is twice as large as that of the  $\alpha 2$  band. These results are consistent with the fact that the known helical structure of Type I collagen is composed of three polypeptide chains  $\alpha 1$ ,  $\alpha 1$  and  $\alpha 2$ .

A simple size-exclusion high performance liquid chromatography (HPLC) method for the separation of collagen monomers and oligomers using a Nucleogel aqua-OH 50-8 column was applied. Two component peaks eluting serially from the column were identified as oligomers and monomers. It

would be expected that oligomers are retained on the size exclusion column much less than monomers. The peak maximum ratio of oligomer to monomer in the collagen samples (a-e) and the standard (f) were, 0.68, 0.77, 0.65, 0.35, 0.62 and 0.34, respectively (Fig. 6). The ratio of the telopeptide-poor collagen designated as H/A (d) was qualitatively identical to that of the standard collagen (f). As is well known, the inherent sensitivity of collagen is also a function of the oligomer content. The higher the oligomer content the more the sensitivity induced [18]. Therefore, collagen samples with a low content of oligomer is considered to be suitable. This form of collagen was used to develop the drug carrier for transdermal delivery.

The viscous nature of collagen solubilized in an acidic solution such as citric acid is considered to be suitable as a transdermal carrier. In addition, the moisturization effect of collagen on the skin might be promising as an enhancer. However, cosolvents are usually necessary for the improvement of drug solubility in the vehicle as well as for the enhancement of drug penetration. The cosolvents commonly used for this purpose include ethanol, isopropyl alcohol, glycerol, and propylene glycol etc. Nevertheless, the denaturation of protein substances, such as collagen, by cosolvents is unavoidable. It would be valuable to understand how much collagen can tolerate these cosolvents in order for successfully employing collagen as a gelling agent in the preparation of a transdermal carrier.

Eleven formulations of collagen films were prepared as shown in Table 1. The results from mechanical strength measurement of these formulations are shown in Table 2. In order to understand the contribution of each vehicle component in the film preparation, the relationship between the mechanical properties of the final product and the compositions was studied through model fitting using the software Design-Expert. The tensile strength was taken as a response and

the components of vehicle as variables. When tensile strength, water, propylene glycol, and ethanol are symbolized as  $R$ ,  $X_1$ ,  $X_2$ , and  $X_3$  accordingly, the relationship can be expressed as a quadratic equation. For the HAP-free collagen film:

$$R = 573.15 \cdot X_1 - 28884.86 \cdot X_2 - 3617.80 \cdot X_3 + 443.52 \cdot X_1 X_2 + 80.03 \cdot X_1 X_3 + 52.93 \cdot X_2 X_3.$$

$$\text{For the HAP-collagen film: } R = 0.089 \cdot X_1 - 0.45 \cdot X_2 + 0.13 \cdot X_3 + 8.341 \cdot E-03 \cdot X_1 X_2 - 4.814 \cdot E-04 \cdot X_1 X_3 + 6.576 \cdot E-03 \cdot X_2 X_3.$$

The existence of HAP weakened the strength of the film, probably due to the destruction of the consistency of the structure. Water appeared to be the most important variable on the mechanical strength of the HAP-free collagen film. For HAP-collagen film, these vehicle components showed less influence on the mechanical strength of the film.

By the examination of X-ray (Fig. 7) and SEM (Fig. 8), it was confirmed that hydroxyapatite was formed inside the collagen film. However, the tensile strength of collagen films without hydroxyapatite was 4-5 time higher than that with hydroxyapatite. This is probably due to the discontinuity of film structure caused by the existence of hydroxyapatite in the collagen films. This results in the easiness of breakage when the force was applied onto the collagen films.

The permeability of proteins penetrated through the collagen films without hydroxyapatite decreased with the increase of molecular weight of protein and with the added amount of propylene glycol in the film formation. There showed a lower flux for the same protein molecule penetrated through the collagen films with hydroxyapatite than that without hydroxyapatite (Table 3 and 4).

The influence of glutaraldehyde on physical properties of type I collagen molecules after crosslinking with different extent was studied by measuring the viscosity change of collagen gel solution, and results are shown

in Figure 9. It shows that the viscosity of collagen gel solutions increased with increasing concentrations of glutaraldehyde. It indicated a gradual transformation of the liquid characteristics of the collagen solution into solid characteristics as the concentration of glutaraldehyde used increased. However, the viscosity of collagen gel solutions reached plateau and became constant when the concentration of glutaraldehyde used was higher than 0.2% at 1% w/w concentration of collagen. These results appear to be in agreement with some literature data, which suggest that glutaraldehyde concentrations are important in determining the nature of the crosslinked network generated [19].

The purpose of using Cremophor RH40 is to enhance the solubility of these hydrophobic model drugs in the collagen solution. The effect of Cremophor RH40 and model drugs (retinoic acid, retinol palmitate, ascorbic acid 6-palmitate and tocopherol acetate) on the viscosity of collagen gel solution was also examined correspondingly and illustrated in Fig. 10. It can be clearly seen that the presence of Cremophor RH40 in collagen gel solution resulted in decreasing the viscosity of collagen solution, but in different extent with simultaneous presence of different model drugs. The extent of reduction in the viscosity of collagen gel solution was also dependent on the concentration of Cremophor RH40. However, there were no significant alternation of viscosity of collagen solution in the presence of each individual model drugs only (Fig. 10A and 10B). Probably, a surfactant such as Cremophor RH40 could play like a lubricant between collagen fibrils to minimize attrition resulting in the decrease of the viscosity of these collagen gel solutions. Expectedly, the viscosity thus decreases with increasing concentration of Cremophor RH40. In term of model drugs (retinoic acid, retinol palmitate, ascorbic acid 6-palmitate, and tocopherol acetate), a minimal interaction between collagen fibrils and these model drugs was suspected due to the hydrophobic nature of these model drugs with a limited

amount of them being solubilized in the collagen solution. As a result, the influence on the viscosity of collagen gel solutions with the presence of these model drugs was observed to be insignificant.

Experiments were conducted to examine the diffusion characteristics of collagen films without crosslinking or crosslinking with different concentration of glutaraldehyde prepared from collagen gel solutions following the procedure described in the Experimental Section. Model drugs, including retinoic acid, retinol palmitate, ascorbic acid 6-palmitate, and tocopherol acetate were prepared either in solution or collagen gel matrix.

The flow-through diffusion cell system can be assumed 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 permeant in the receiver cell,  $A$  is the diffusion area,  $J(t)$  is input rate from 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

$$C(t) = 0, \quad \text{when } t = 0 \quad (2)$$

The area term was dropped from the following equations assuming unit diffusion area. Depending on  $J(t)$ , flux, the solution to Eq. 1 can be different. At steady-state condition,  $J(t) = J_{ss}$ , Eqs 1 and 2 can be solved for  $C(t)$  to

$$C(t) = \frac{J_{ss}}{F_0} - \frac{J_{ss}}{F_0} \exp\left(-\frac{F_0 t}{V}\right) \quad (3)$$

Eq. 3 describes the concentration profile of permeant in the receiver cell as a function of time. The concentration in the receiver cell

increases with time and approaches the plateau value of  $J_{ss}/F_0$  as time approaches infinity [11]. Then, Eq. 3 is simplified to

$$C(t) = \frac{J_{ss}}{F_0} \quad (4)$$

According to Fick's law, the steady state flux ( $J_{ss}$ ) is constant and is expressed by:

$$J_{ss} = \frac{DK}{h}(C_d - C_r) \quad (5)$$

where  $D$  and  $K$  are the diffusion and-partition coefficients,  $C_d$  and  $C_r$  are the concentrations of solutes in the donor cell and receptor cell, respectively. The value of  $J_{ss}$  was calculated from the slope of linear plot of cumulative amount of permeant versus time, which are equivalent to that obtained by Eq. 5. 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. 5 with that  $h$  was designated as the thickness of collagen films.

The values of steady state flux ( $J_{ss}$ ) of model drugs through collagen films were obtained from the slopes of the plots in Fig. 11 based on Eqs. 4 and 5 and results were summarized in Table 5. Plots of the values of steady state flux ( $J_{ss}$ ) of solution and collagen gel form of model drugs vs glutaraldehyde concentration were also shown in Fig. 12. The values of  $J_{ss}$  decreases with increasing concentration of glutaraldehyde for those model drugs of retinol palmitate, ascorbic acid 6-palmitate and tocopherol acetate for both solution and collagen matrix. Furthermore, these values of  $J_{ss}$  approached a constant when the concentration of glutaraldehyde used for crosslinking exceeded 0.2%. These results demonstrated that the closeness between viscosity change

of collagen solution in the presence of different concentration of glutaraldehyde and diffusion characteristics of model drugs through collagen film treated with glutaraldehyde.

Nevertheless, a significant increase in the values of  $J_{ss}$  of retinoic acid through collagen film was noticed when the concentration of glutaraldehyde used for crosslinking was higher than 0.25%. Since the permeability is defined as the ratio of product of the partition coefficient and the diffusion coefficient to the thickness of film. It would be more appropriate to examine the separate effects of partitioning and diffusion behavior. Table 6 lists the permeability, partition and diffusion coefficients of retinoic acid through collagen films treated with glutaraldehyde by different concentration. Permeability was calculated by dividing  $J_{ss}$  with the solubility of retinoic acid in the donor compartment. With known partition coefficients and the thickness of collagen films, diffusion coefficients were determined accordingly. The variability of permeability, partition coefficient, and diffusion coefficient for retinoic acid through collagen films treated with different concentration of glutaraldehyde were shown in Fig. 13. There is a significant reduction in partition coefficients for collagen film crosslinked with various concentration of glutaraldehyde compared to collagen film with no crosslinking. A tendency of decreasing partition coefficient with increasing the extent of crosslinking was further observed. However, the extent of reduction was not as large as that between treated and untreated collagen films. Also, the decrease of diffusion coefficient with increasing the extent of crosslinking of collagen films was demonstrated initially, but then increase for those collagen films crosslinked with the concentration of glutaraldehyde exceeding 0.2%.

Actually, the introduction of hydrophobic nature into collagen films by crosslinking with glutaraldehyde was expectable since

two aldehyde groups of glutaraldehyde are connected by a hydrophobic alkyl chain. Therefore, the increase of partition coefficients into collagen films for a hydrophobic compound with no interaction was expectable with increasing the extent of crosslinking by introducing an increasing amount of glutaraldehyde. Hydrophilicity of collagen fibrils was further reduced as a result that most of hydrophilic  $\epsilon$ -amine group of lysine residuals was crosslinked with a hydrophobic crosslinker of glutaraldehyde. The decreasing percentage of free  $\epsilon$ -amine groups with increasing concentration glutaraldehyde for crosslinking has been demonstrated (data not shown). This result will conflict with what has been observed that a decrease of partition coefficient for retinoic acid into collagen films with increasing the extent of crosslinking. A better explanation for this might be that there exists an interaction between the carboxylic group of retinoic acid and  $\epsilon$ -amine group of lysine residuals. It would be less favorable for retinoic acid to partition into collagen films with free  $\epsilon$ -amine groups in collagen films becoming exhausted. Therefore, increasing hydrophobicity and less favorable interaction with increasing the extent of crosslinking make partition coefficients of retinoic acid into collagen films crosslinked with different concentration of glutaraldehyde showing a comparable value as shown in Fig. 13.

Scanning electric microscopy (SEM) also demonstrated that the pore size of collagen films decreased with increasing the extent of crosslinking. It would expect a more rigid structure and entanglement of the fibrils in those collagen films treated with glutaraldehyde than that of untreated one, resulting in the decrease of permeability and diffusion coefficients. Nevertheless, the permeability and diffusion coefficients of retinoic acid decreased correspondingly with increasing the extent of crosslinking, and then increased as the extent of crosslinking exceeding 90%. These phenomena might be a result that complete crosslinking of terminal amino groups on collagen was

approached when the concentration of glutaraldehyde used was higher than 0.25%. 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 film [19]. The extent of interaction during diffusion process between crosslinked collagen film and retinoic acid seems to decrease with increasing the concentration of glutaraldehyde. As a result, retinoic acid permeates easily through collagen films crosslinked with higher concentration of glutaraldehyde.

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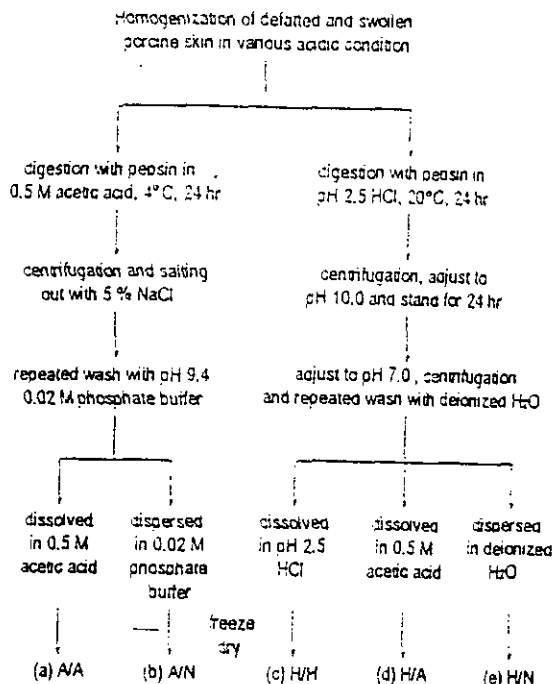


Fig. 1. Flow chart for various methods of purification of collagen from porcine skin.

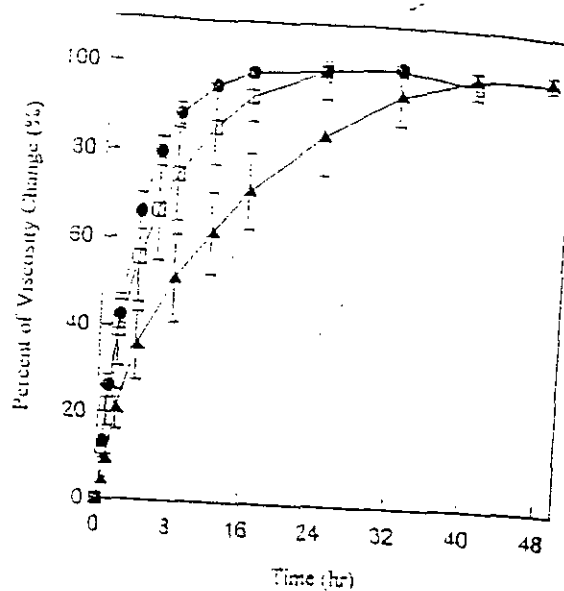


Fig. 2. The percent viscosity change of collagen homogenate digested by various ratios of pepsin to skin (●, 1:50; □, 1:100; ▲, 1:200) in pH 2.5 HCl, 25°C ( $n = 3$ ).

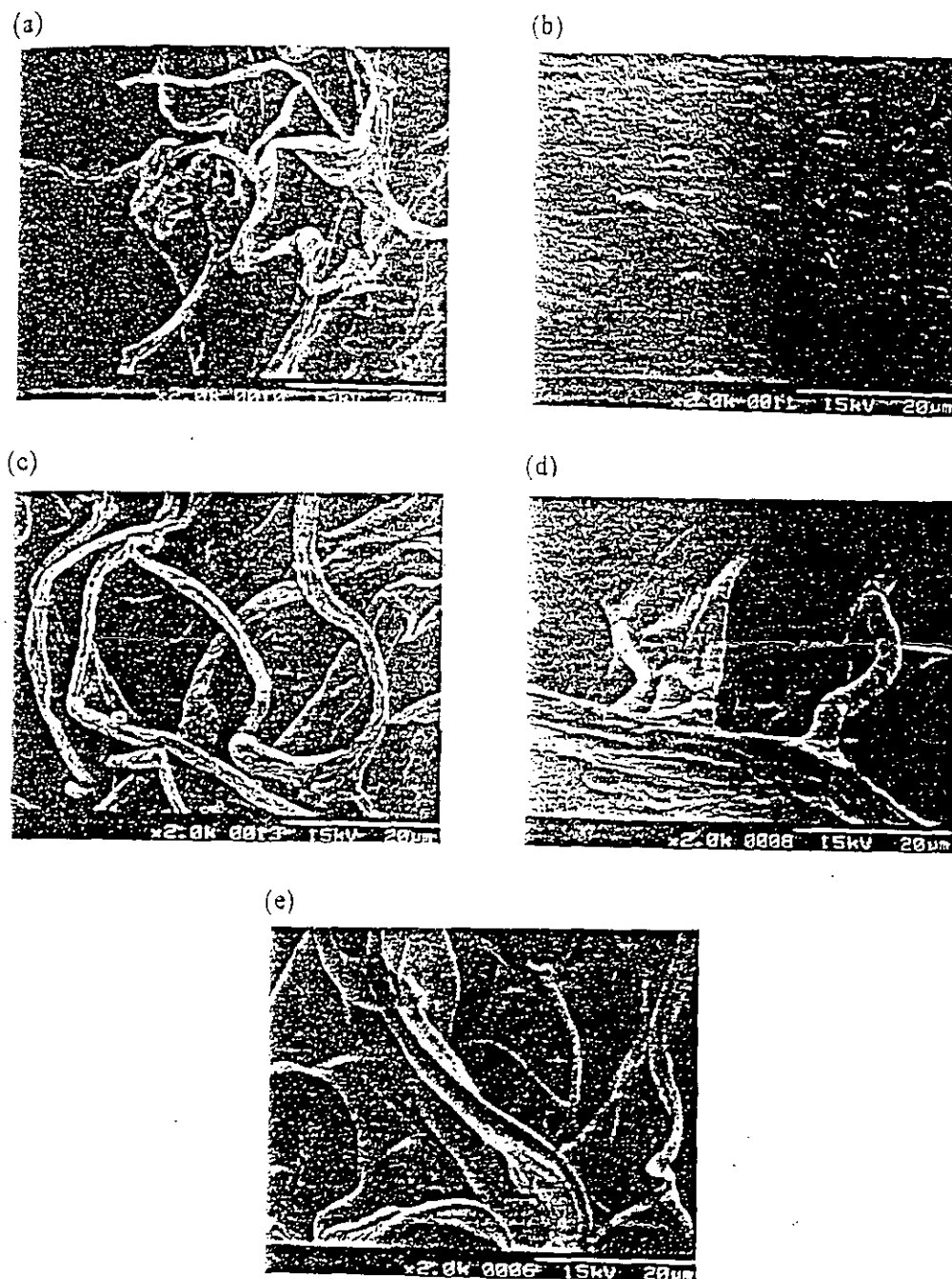


Fig. 3. SEM of various preparations of collagen (15 KV X2000). (a) A/A (b) A/N (c) H/H (d) H/A (e) H/N

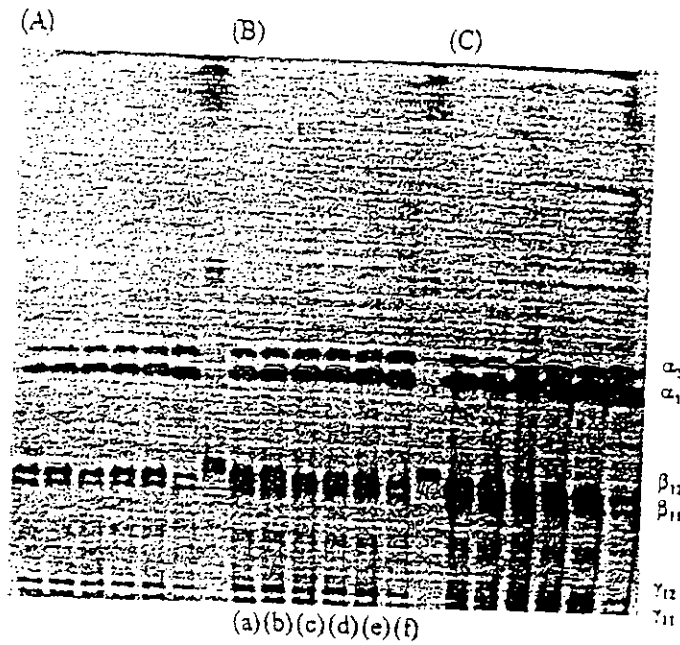


Fig. 4. SDS-PAGE electrophoresis of various preparations of collagen, stained with Coomassie blue R-250. Loading amounts: (A) 5  $\mu$ g, (B) 10  $\mu$ g and (C) 20  $\mu$ g. Key: (a) A/A, (b) A/N, (c) H/H, (d) H/A, (e) H/N and (f) standard.

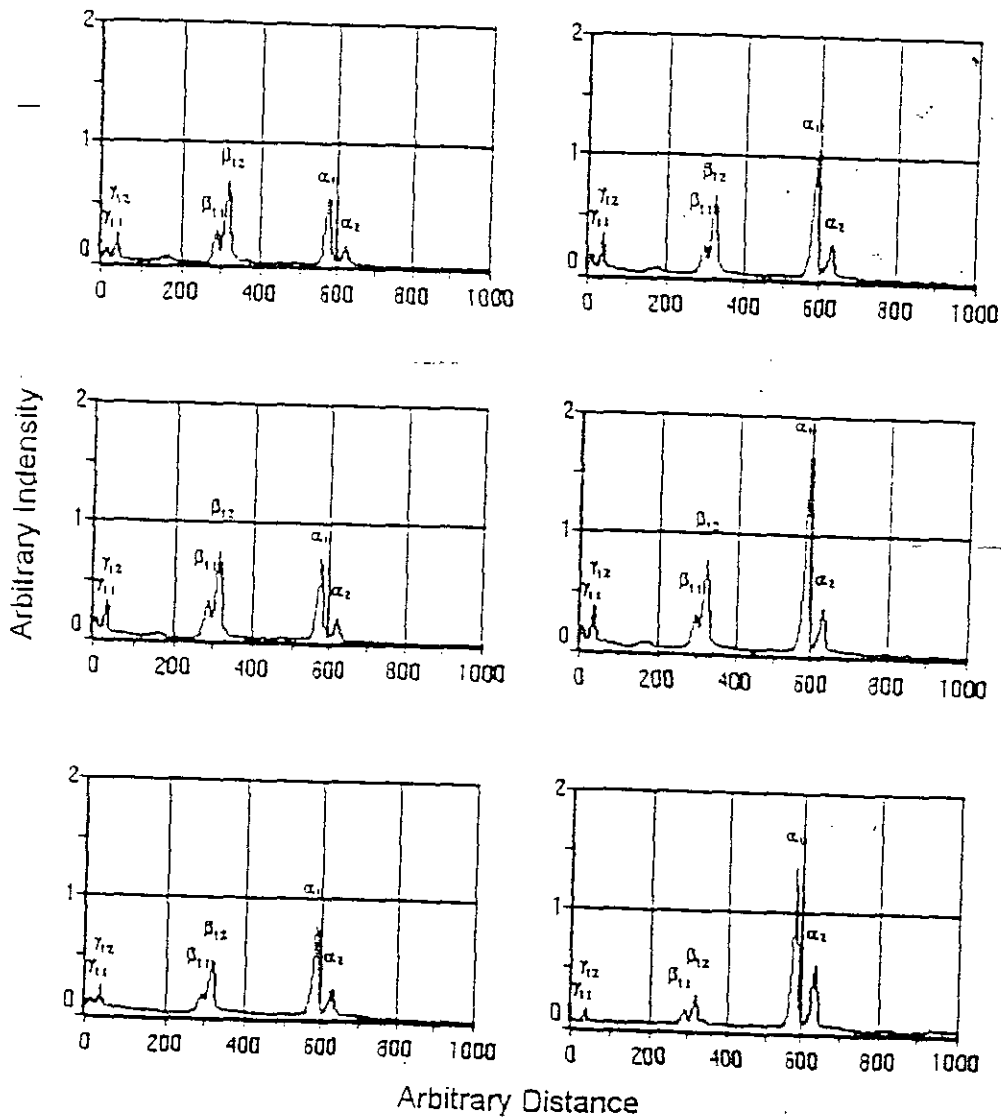


Fig. 5. One-dimensional densitograms of SDS-PAGE electrophoresis for various preparation of collagen. (a) A/A, (b) A/N, (c) H/H, (d) H/A, (e) H/N and (f) standard.

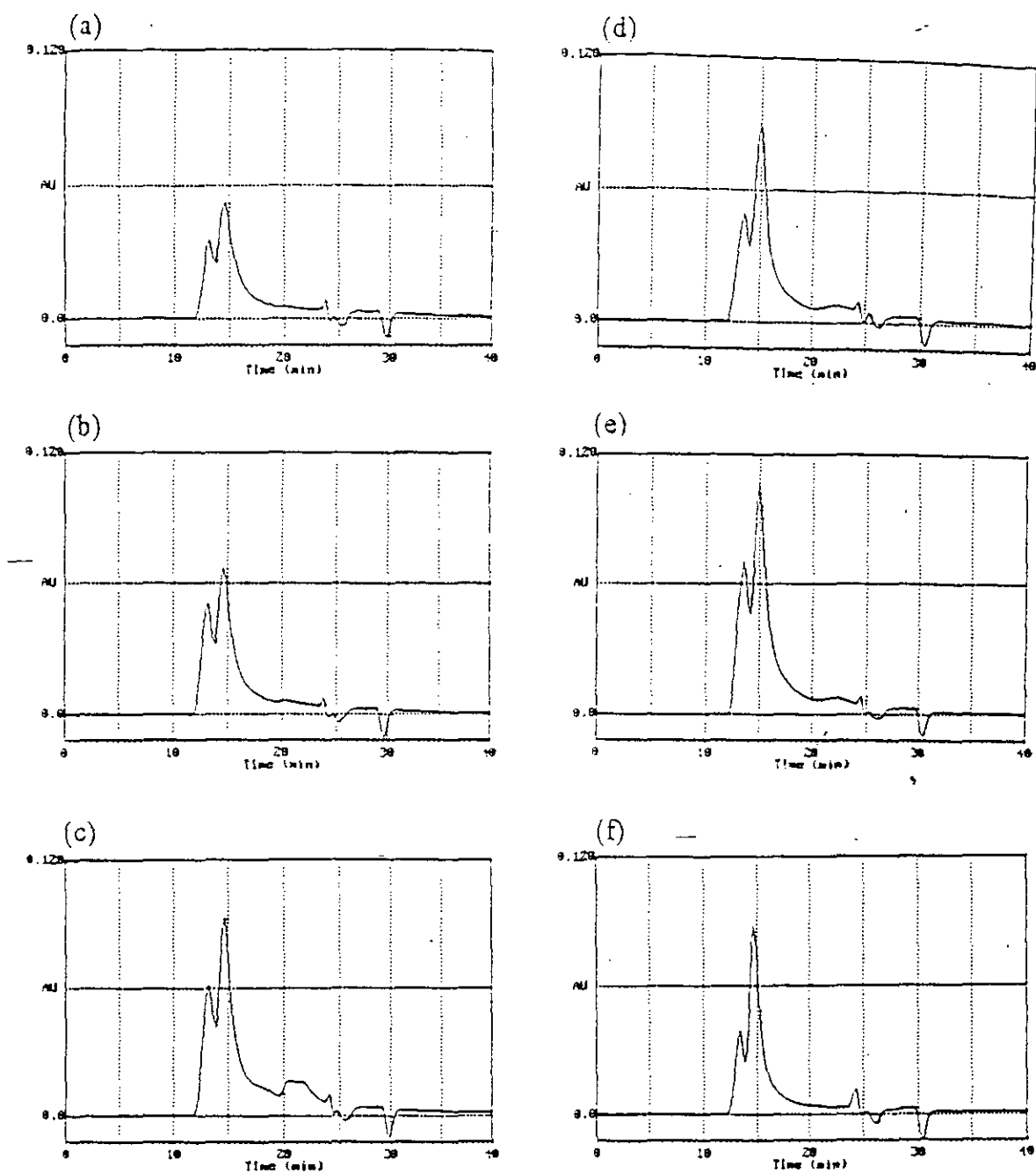


Fig. 6. Size-exclusion chromatography of various preparations of collagen.-Peak maximum ratio = (oligomer/monomer). (a) A/A, 0.68; (b) A/N, 0.77; (c) H/H, 0.65; (d) H/A, 0.35; (e) H/N, 0.62; (f) standard, 0.34.

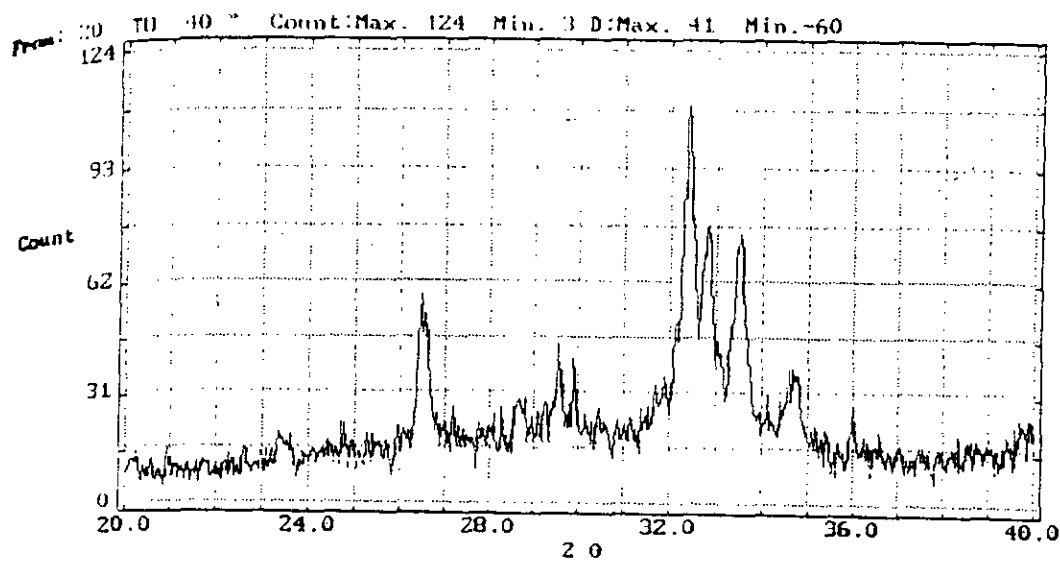


圖 7 、在 collagen film 的存在下合成的

hydroxypatite X-ray 圖譜

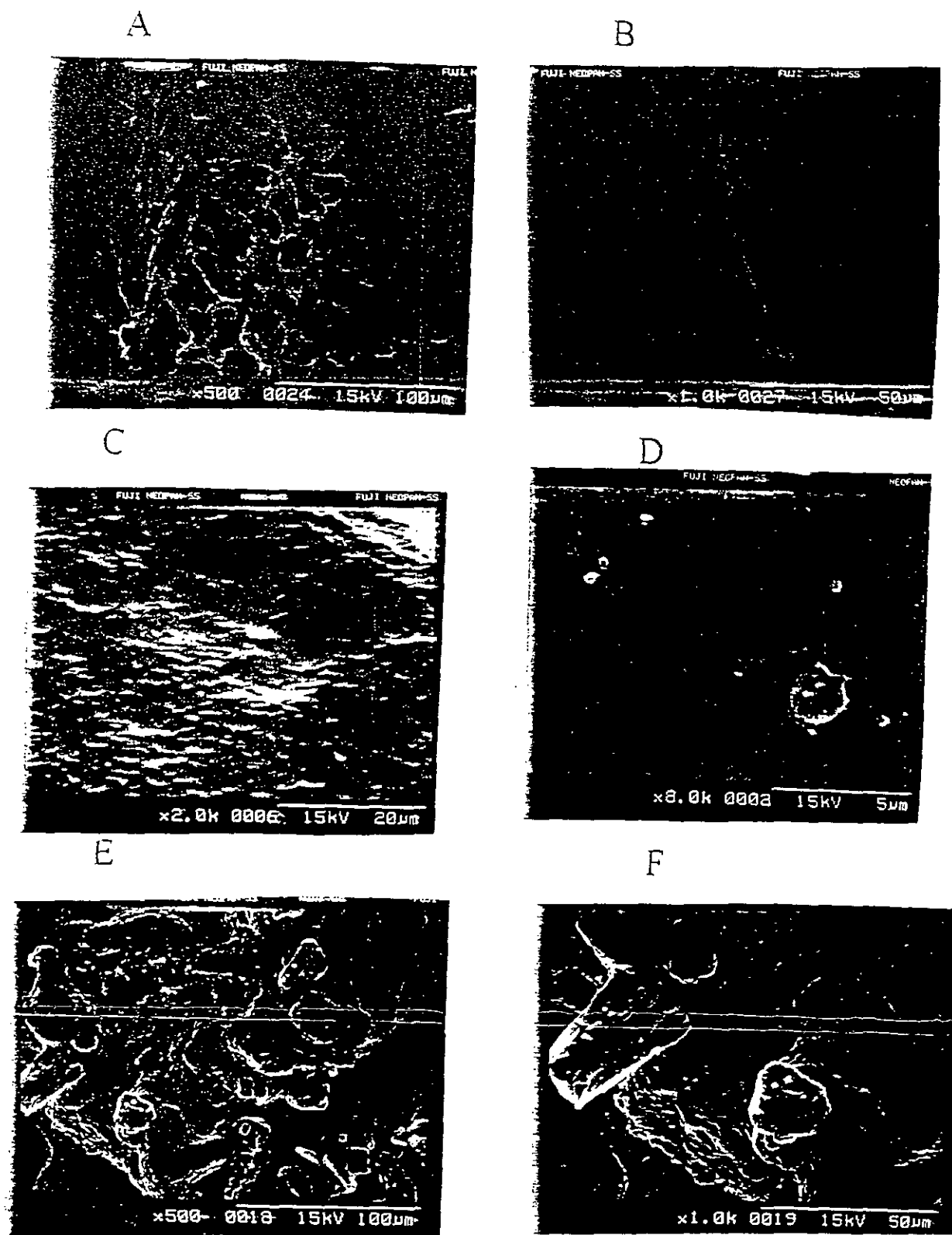
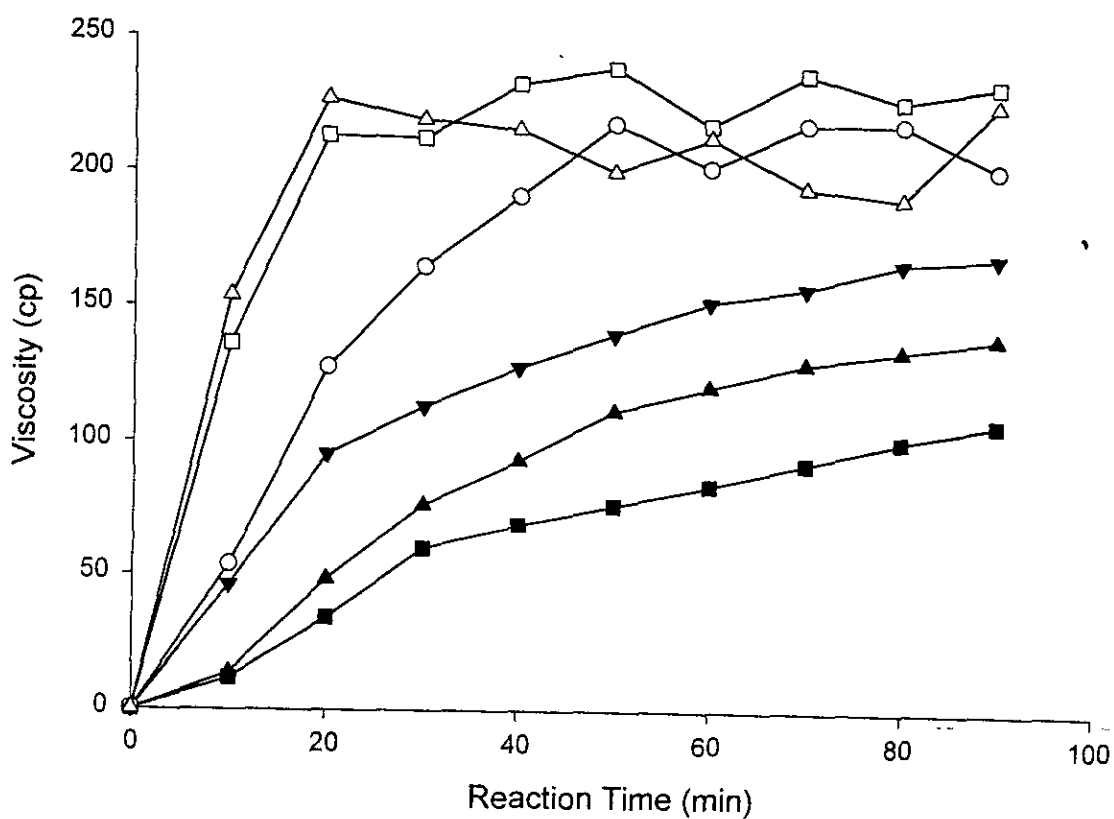
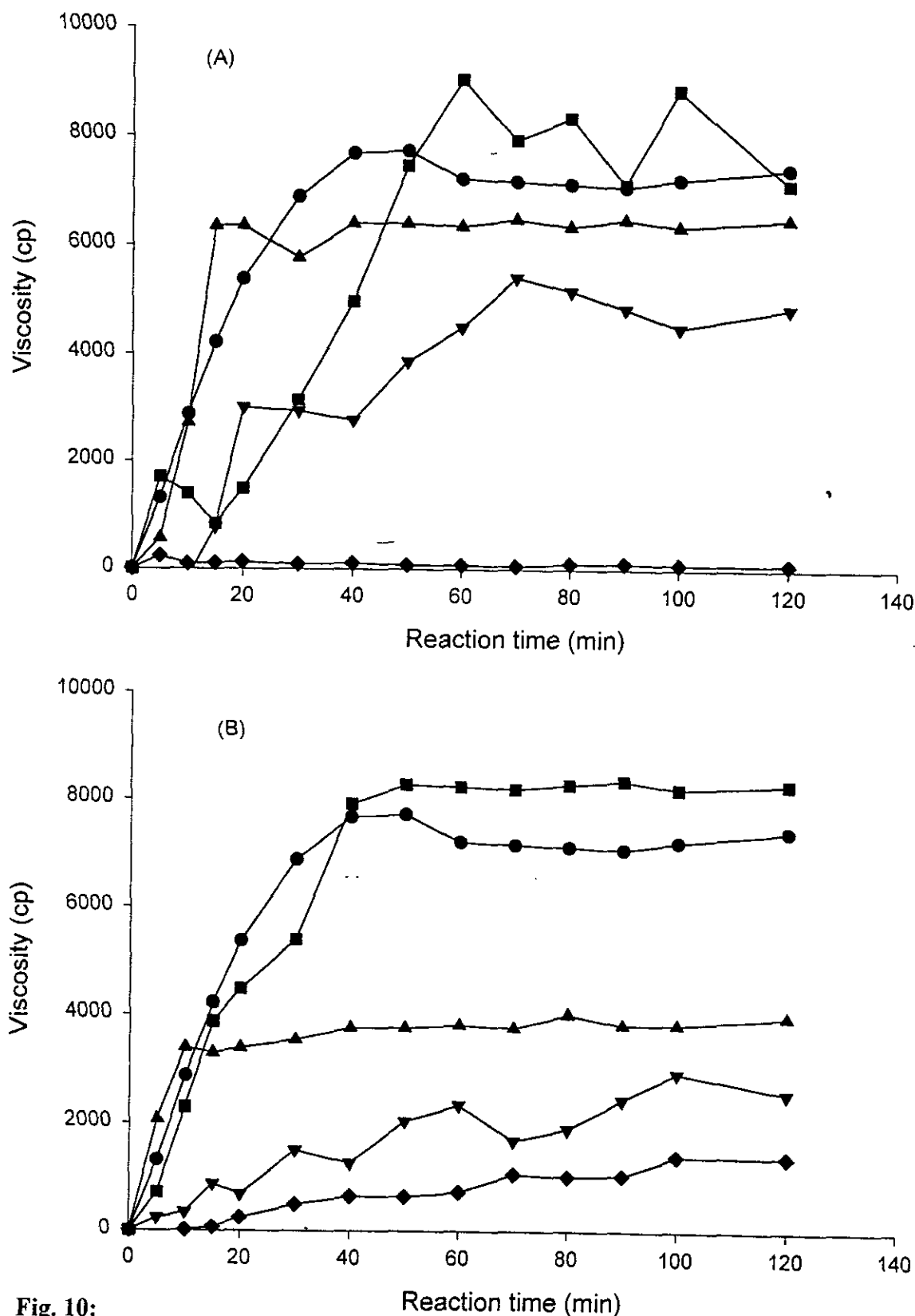


圖 8 、 A 、 B collagen sponge ; C 膠原蛋白膜穿  
透前 ; D 穿透後 ; E 、 F 膠原蛋白膜中的 hydroxyapatite



**Fig. 9:**

The effect of different concentration of glutaraldehyde on the viscosity change of collagen gel solution (■, 0.05%; ▲, 0.075%; ▼, 0.1%; ○, 0.2%; □, 0.25%; △, 0.3%).



**Fig. 10:**

The effect of Cremophor RH40 and model drugs ((A): retinoic acid, (B): retinol palmitate + ascorbic acid 6-palmitate + tocopherol acetate) on the viscosity change of collagen gel solution (●: collagen only; ■: collagen + model drug; ▲: collagen + model drug + Cremophor RH40 (1%); ▼: collagen + model drug + Cremophor RH40 (5%); ◆: collagen + model drug + Cremophor RH40 (10%)).

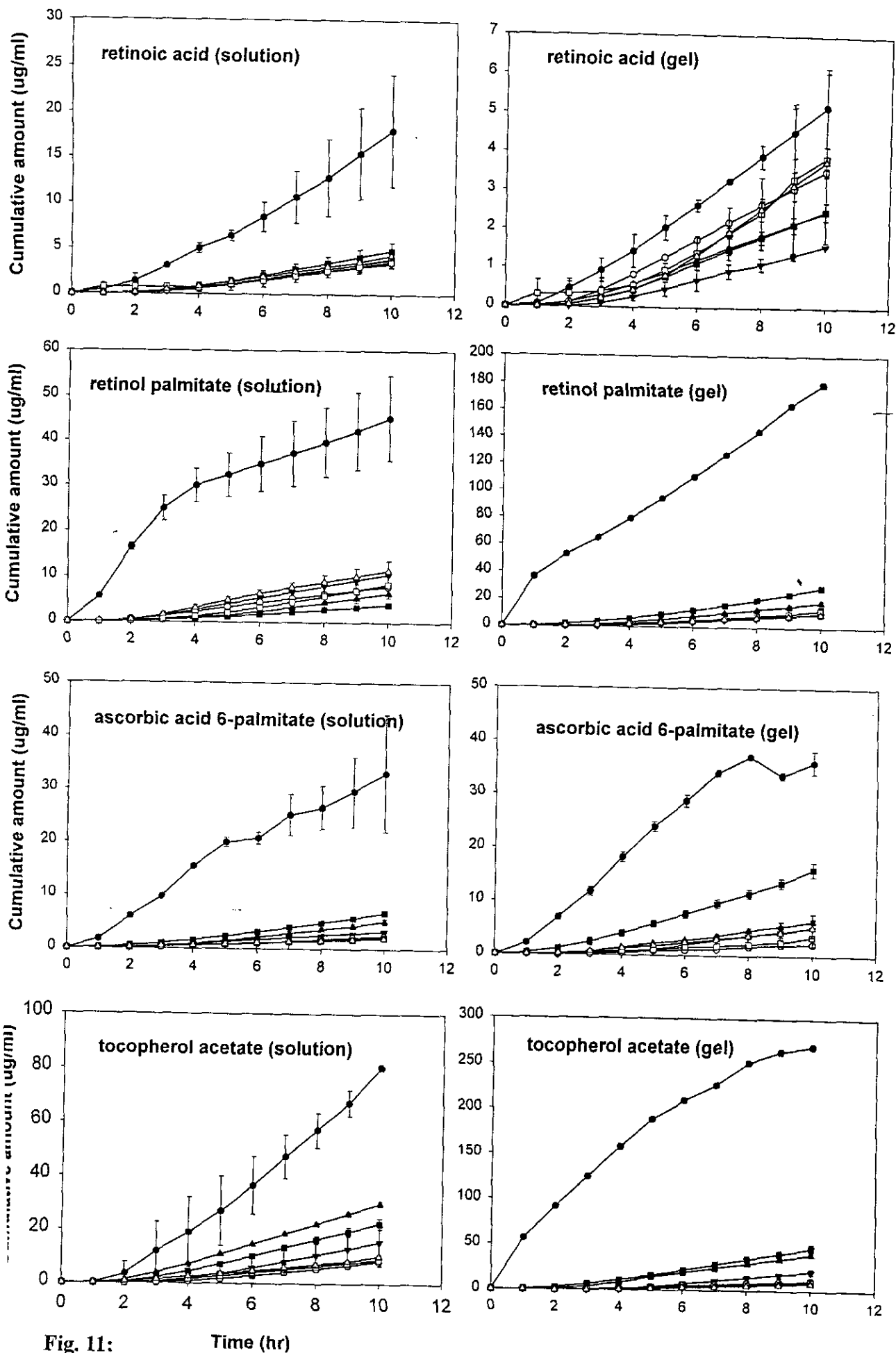
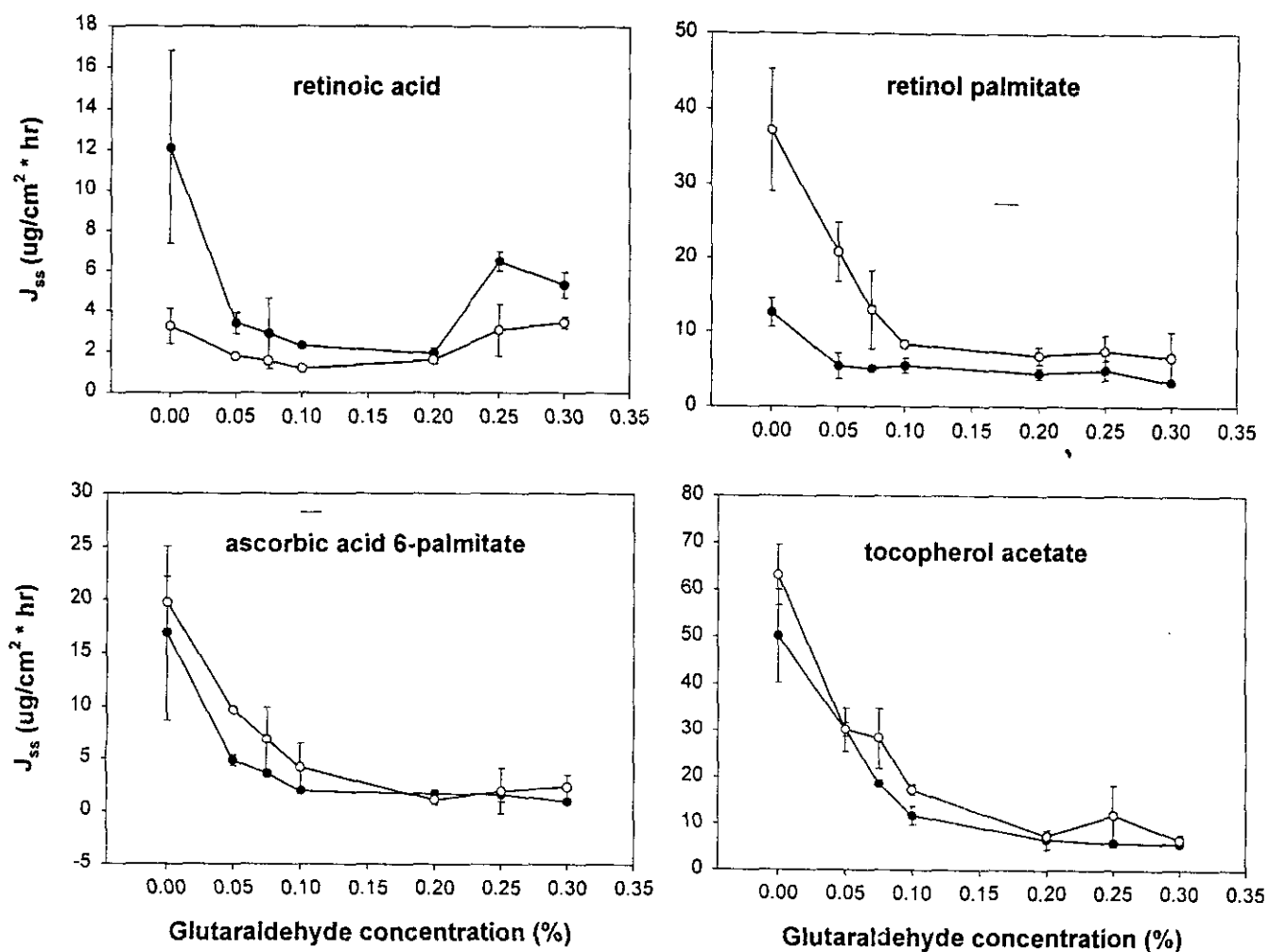


Fig. 11:

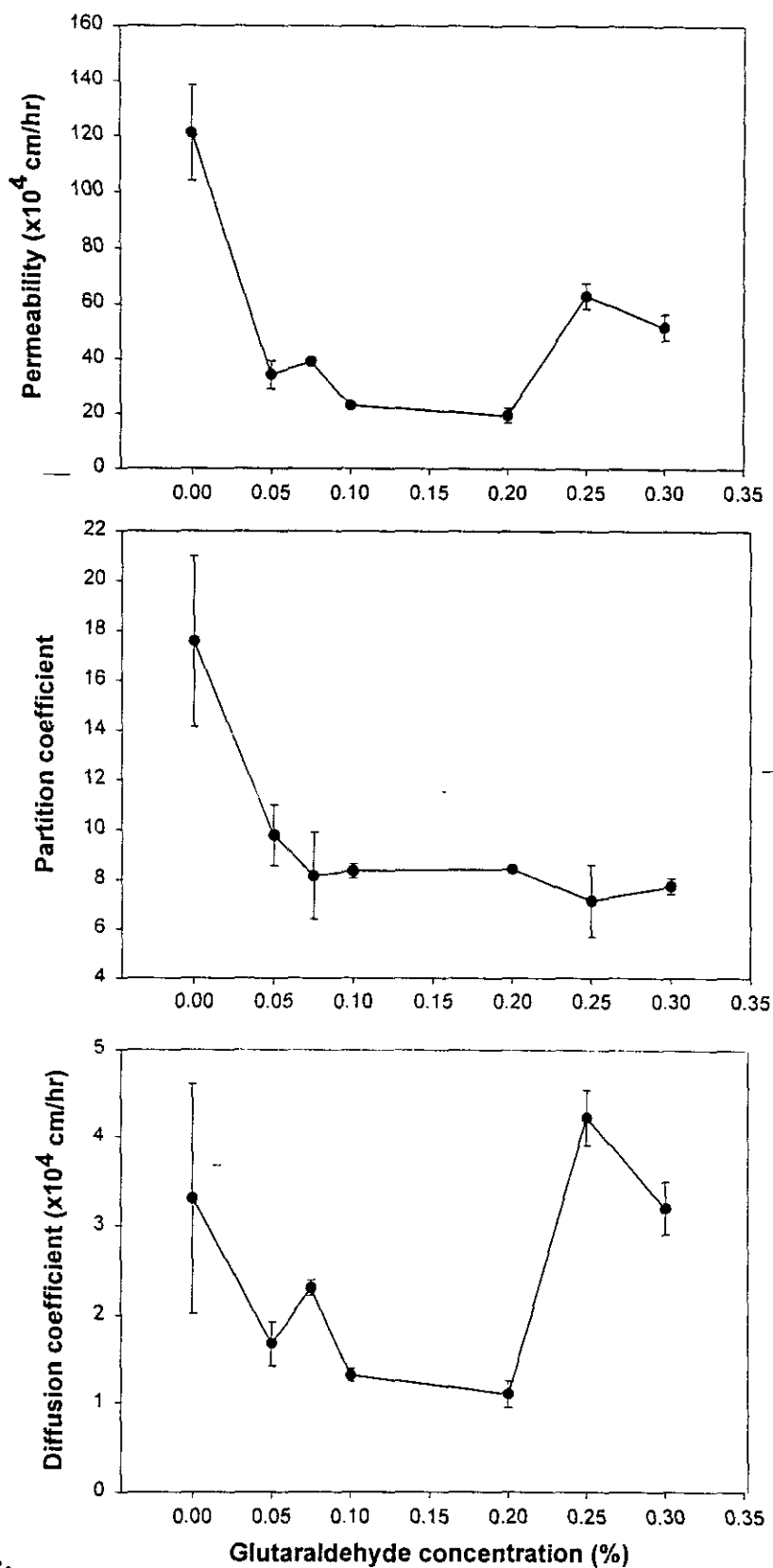
Time (hr)

The cumulative amount of model drugs penetrated versus time from either solution or gel form through collagen films treated with different concentration of glutaraldehyde (●, 0%; ■, 0.05%; ▲, 0.075%; ▼, 0.1%; ○, 0.2%; □, 0.25%; △, 0.3%)



**Fig. 12:**

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



**Fig. 13:**

The variability of permeability, partition coefficient, and diffusion coefficient for retinoic acid through collagen films treated with different concentration of glutaraldehyde

表 1 膠原蛋白膜的處方設計

|                | <i>H<sub>2</sub>O</i><br>(%) | <i>Propylene glycol</i><br>(%) | <i>Alcohol</i><br>(%) |
|----------------|------------------------------|--------------------------------|-----------------------|
| Formulation 1  | 78.33                        | 13.33                          | 8.33                  |
| Formulation 2  | 95                           | 5                              | 0                     |
| Formulation 3  | 74.17                        | 9.17                           | 16.67                 |
| Formulation 4  | 70                           | 30                             | 0                     |
| Formulation 5  | 70                           | 17.5                           | -12.5                 |
| Formulation 6  | 70                           | 5                              | 25                    |
| Formulation 7  | 86.67                        | 9.17                           | 4.17                  |
| Formulation 8  | 82.5                         | 5                              | 12.5                  |
| Formulation 9  | 74.17                        | 21.67                          | 4.17                  |
| Formulation 10 | 82.5                         | 5                              | 12.5                  |
| Formulation 11 | 82.5                         | 17.5                           | 0                     |

表 2 、膠原蛋白膜的彈張力

|                | Stress(mN/cm <sup>2</sup> ) |          | Strain(%) |          | Modulus(psi) |          |
|----------------|-----------------------------|----------|-----------|----------|--------------|----------|
|                | A film*                     | B film** | A film*   | B film** | A film*      | B film** |
| Formulation 1  | 155238                      | 29787    | 50.57     | 92.98    | 306.9765     | 32.03592 |
| Formulation 2  | 122574                      | 23548    | 48.79     | 50.12    | 251.2277     | 46.98324 |
| Formulation 3  | 135877                      | 83703    | 51.24     | 67.94    | 265.1776     | 123.2014 |
| Formulation 4  | 105291                      | 29133    | 54.91     | 60.13    | 191.752      | 48.45002 |
| Formulation 5  | 109577                      | 70526    | 44.36     | 77.42    | 247.0176     | 91.09532 |
| Formulation 6  | 105679                      | 21357    | 49.54     | 50.44    | 213.3205     | 42.3414  |
| Formulation 7  | 142859                      | 85102    | 55.13     | 45.61    | 259.1311     | 186.5863 |
| Formulation 8  | 1152917                     | 239714   | 132.86    | 55.33    | 867.7683     | 433.2442 |
| Formulation 9  | 142946                      | 23722    | 50.43     | 66.34    | 283.4543     | 35.75822 |
| Formulation 10 | 125614                      | 20567    | 58.16     | 71.23    | 215.9801     | 28.87407 |
| Formulation 11 | 184088                      | 100421   | 50.47     | 81.77    | 364.7474     | 122.8091 |

\*Collagen film : \*\*collagen film with 10mM hydroxyapatite

表3、Proteins 在膠原蛋白膜(不含 hydroxyapatite)的穿透

|   | H <sub>2</sub> O<br>(%) | Propylene<br>glycol<br>(%) | Alcohol<br>(%) | Lysozyme                                  |                                           | Carbonic anhydrase                        |                                           | Albumin, Egg                              |                                           | Albumin, bovine                           |                                           |
|---|-------------------------|----------------------------|----------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|
|   |                         |                            |                | Permeability<br>(cm/min)( $\times 10^5$ ) | Permeability<br>(cm/min)( $\times 10^5$ ) | Permeability<br>(cm/min)( $\times 10^6$ ) | Permeability<br>(cm/min)( $\times 10^6$ ) | Permeability<br>(cm/min)( $\times 10^7$ ) | Permeability<br>(cm/min)( $\times 10^7$ ) | Permeability<br>(cm/min)( $\times 10^7$ ) | Permeability<br>(cm/min)( $\times 10^7$ ) |
| 1 | 78.33                   | 13.33                      | 8.33           | 3.94 $\pm$ 0.11*                          | 3.55 $\pm$ 0.31                           | 4.31 $\pm$ 0.01                           | 1.89 $\pm$ 0.02                           |                                           |                                           |                                           |                                           |
| 3 | 74.17                   | 9.17                       | 16.67          | 5.79 $\pm$ 0.13                           | 4.79 $\pm$ 0.02                           | 5.78 $\pm$ 0.02                           | 3.59 $\pm$ 0.01                           |                                           |                                           |                                           |                                           |
| 5 | 70.00                   | 17.50                      | 12.5           | 2.14 $\pm$ 0.06                           | 2.51 $\pm$ 0.12                           | 2.59 $\pm$ 0.01                           | 1.02 $\pm$ 0.02                           |                                           |                                           |                                           |                                           |
| 8 | 82.50                   | 5.00                       | 12.50          | 6.97 $\pm$ 0.02                           | 5.67 $\pm$ 0.15                           | 9.12 $\pm$ 0.02                           | 7.52 $\pm$ 0.01                           |                                           |                                           |                                           |                                           |

\*: Mean $\pm$ Standard Error

表4、Proteins 在膠原蛋白膜(包含 hydroxyapatite)的穿透

|   | H <sub>2</sub> O<br>(%) | Propylene<br>glycol<br>(%) | Alcohol<br>(%) | Lysozyme                                  |                                           | Carbonic anhydrase                        |                                           | Albumin, Egg                              |                                           | Albumin, bovine                           |                                           |
|---|-------------------------|----------------------------|----------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------------------|
|   |                         |                            |                | Permeability<br>(cm/min)( $\times 10^5$ ) | Permeability<br>(cm/min)( $\times 10^5$ ) | Permeability<br>(cm/min)( $\times 10^7$ ) | Permeability<br>(cm/min)( $\times 10^7$ ) | Permeability<br>(cm/min)( $\times 10^5$ ) | Permeability<br>(cm/min)( $\times 10^5$ ) | Permeability<br>(cm/min)( $\times 10^8$ ) | Permeability<br>(cm/min)( $\times 10^8$ ) |
| 1 | 78.33                   | 13.33                      | 8.33           | 4.19 $\pm$ 0.12*                          | 3.32 $\pm$ 0.40                           | 3.81 $\pm$ 0.01                           | 4.39 $\pm$ 0.01                           |                                           |                                           |                                           |                                           |
| 3 | 74.17                   | 9.17                       | 16.67          | 5.77 $\pm$ 0.23                           | 4.15 $\pm$ 0.42                           | 7.62 $\pm$ 0.02                           | 4.77 $\pm$ 0.01                           |                                           |                                           |                                           |                                           |
| 5 | 70.00                   | 17.50                      | 12.5           | 3.66 $\pm$ 0.08                           | 2.93 $\pm$ 0.41                           | 2.11 $\pm$ 0.03                           | N.D.                                      |                                           |                                           |                                           |                                           |
| 8 | 82.50                   | 5.00                       | 12.50          | 7.43 $\pm$ 0.03                           | 4.46 $\pm$ 0.21                           | 9.51 $\pm$ 0.02                           | 6.48 $\pm$ 0.01                           |                                           |                                           |                                           |                                           |

\*: Mean $\pm$ Standard Error

Table 5: The values of  $J_{ss}$  ( $\mu\text{g}/\text{cm}^2\cdot\text{hr}$ ) for model drugs across collagen films crosslinked with different concentration of glutaraldehyde

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

\*: Mean  $\pm$  standard error

Table 6: Permeability, partition and diffusion coefficients for retinoic acid across collagen films crosslinked with different concentrations of glutaraldehyde

| Glutaraldehyde (%) | Permeability (cm <sup>2</sup> /hr) (×10 <sup>4</sup> ) | Partition coefficient | Diffusion coefficient (cm <sup>2</sup> /hr) (×10 <sup>4</sup> ) |
|--------------------|--------------------------------------------------------|-----------------------|-----------------------------------------------------------------|
| 0                  | 121.17±17.30*                                          | 17.56±3.43            | 3.31±1.29                                                       |
| 0.05               | 34.31±5.15                                             | 9.78±1.22             | 1.68±0.25                                                       |
| 0.075              | 39.12±1.45                                             | 8.15±1.74             | 2.30±0.08                                                       |
| 0.1                | 23.09±1.33                                             | 8.36±0.30             | 1.32±0.07                                                       |
| 0.2                | 19.43±2.69                                             | 8.44±0.08             | 1.11±0.15                                                       |
| 0.25               | 63.06±4.67                                             | 7.16±1.46             | 4.22±0.31                                                       |
| 0.3                | 51.91±4.77                                             | 7.77±0.325            | 3.21±0.29                                                       |

\*Mean ± standard error

## 出席國際會議報告

### 第二十七屆生體活性物質控釋國際研討會

許明照

臺北醫學院 藥學研究所

#### (一) 參加會議經過

本年度生體活性物質控釋國際研討會為第二十七屆，會議地點轉到法國的巴黎 (Paris)。今年與會人數約在一千五百人左右，參加國家也有三十餘國，口頭發表的論文是以邀請的演講者為主，壁報論文有近四百四十篇左右，更有眾多的廠商參予展覽，人潮鼎盛，盛況是每年都在增強中。正式會議是從七月九日至十三日在巴黎的 pPalais des Congres, Porte Maillot 中進行，提供與會者學習機會，收穫可說頗為豐富。

八日下午開始報到並以 soapbox 的方式為正式大會熱身，晚上舉行歡迎會，為與會人員洗塵並加強人員之間的交流與認識。九日上午以大會演講揭開序幕，首先由 Dr. D. Dormont 由分子生物學與病理學觀點暢談 Prion diseases，Dr. Dormont 對主題的歷史發展與未來展望，以一小時的流暢演講帶出來一個完整輪廓，對於欲探觀此領域的學者有相當的啟發作用，也提供與會者另一完全新穎的觀念。接著頒發年度的論文獎以及獎助研究生參加研討會的名種獎項。很榮幸的，本實驗室參加論文發表的研究生得到免交研討費及部分生活費補貼的獎助，對研究生的參與國際會議有蠻大的鼓勵作用。

接下來的二天半期間，大會仍以小型研討會方式進行多項不同主題的探討與研究發表。重要主題包括：基因療法與寡核醣輸送、微粒輸送系統、疫苗處方、口服輸送、組織工程與細胞層次治療、DNA 輸送、新高分子材質與生醫材料、經皮吸收傳送 (I & II)、藥物標的與特定部位輸送、蛋白質輸送，前驅藥與估分子性藥物、疫苗的小型研討會、藥物控釋用之新材質與載體的小型研討會等。此些內容已涵蓋至今在藥物治療領域中重要的研究主題，為與會者提供最新最先端的研究方向與可能的應用性，也讓與會依自體之興趣選擇合適的研討會與來自世界各地之研究者或專家共聚一堂，共同討論以吸收新知。感觸頗深的是，

此種接觸也讓自己能有更多的啓發與激勵，擴大學術見解，瞭解不進則退的道理。

## (二) 與會心得

琳琅滿目的主題，在時間分配上多有重覆，無法一一參與，只能就以下主題說明其研究近況及與會心得：

### (1) 固態腫瘤之治療 (Solid Tumor Therapy)

癌症治療仍是不間斷的研究主題，合成性藥物的抗癌效果之篩選仍然也是主力的開發方向，但漸漸的發現如何由最根本的基礎研究來瞭解傳統抗癌藥的治療機轉，才是徹底解決如何選擇性的殺死癌細胞，且能避免人體細胞或 viable 細胞的過度死亡之最基本方向。Dr. Ringsdorf 就此探討下一個世紀在高分子型治療劑與超大型分子系統之發展，並說明兩者是未來固體腫瘤治療的主流之一。Dr. Jain 提出生理功能在選擇性毀損癌細胞之治療策略中所扮演的角色。這觀念的研究結果，讓我們深入了解選擇性的破壞癌細胞之血管系統之所以失敗的可能原因，如將已插入一老鼠 IFN- $\gamma$  基因的神經瘤細胞生長在裸鼠身上，因此癌細胞所分泌的 IFN- $\gamma$  將引發癌細胞的血管上皮組織 MHC Class II 的抗原，此種抗原不存在於正常組織的血管上皮結構中，因而其抗體將可選擇性的將與其複合或鍵結的毒素帶至癌細胞的上皮組織上，如果此毒素是如 ricin A-chain 類，將會破壞癌細胞的上皮組織，並在受損的血管內引發血小板的凝聚和形成血栓。此毒素也可以是由基因工程所合成的導引凝血蛋白質如 Tissue Factor 等。但研究的結果發現此類的治療方式在早先幾天真得如所預期可減緩腫瘤的增生而萎縮，但到第五天之後，則又是另一個故事的演變，似乎腫瘤的活性又再度揮復。當深入了解其原因才發現，腫瘤附近的正常細胞所分泌的 VEGF 幫助腫瘤細胞揮復血管系統的生長而重獲活性。除此之外，體外試驗或動物體試驗的微細環境狀態也不同於腫瘤於人體內，而微細環境狀態對腫瘤生長也有不同的影響，此類種種都是在腫瘤治療時應考慮的生理因素；甚至以前從未考慮的氧壓對腫瘤細胞治療的影響，都讓此領域的研究者對腫瘤治療又有另一層的認知。

### (2) 基因治療與疫苗傳送 (Gene Therapy and Vaccine Delivery)

這些年來，基因治療仍維持為熱門的研究題目之一。而類似的疫苗傳送也漸為研究者所熱衷，尤其是以 DNA 為疫苗之抗原體者更與基因傳送有異曲同工之效。基因治療的載體仍以微脂粒為主體，而相對的疫苗輸送則由 viral 的

載體擴大至利用如 Lipoprotein 與 Chitosan 之類的 non-viral 材質，甚至可採用非注射型的口服方式。以裸露型 DNA 為輸送原的基因治療，傳統上是將 DNA 壓縮進入非凝集型的非 viral 粒子，其大小約相似於病毒體。存在於此中的 DNA 仍具有生物活性，而能夠在細胞內表現出外來基因。但目前 non-viral 的載體的發展，則以配套能夠啟動特定轉送的官能基為主，此機轉是應用已完整建立的大型分子或病毒體標的於細胞之不同部位之知識。在解決轉送障礙的研究方面也有相當的突進，利用合成型載體如 polyethylenimine (PEI) 在酸鹼值於 5 至 7 具有緩衝能力，因此可在細胞內因 "proton sponge" 的效用而將 DNA plasmid 解離而達到輸送之目的。在突進最大障礙層的核膜方面也有可行的方法，將 single nuclear localization signal peptide 共價鍵的結合於 reporter 基因可以增加基因表現能力達十倍以上。這都以增加基因治療的成功率，而此技術或許可應用於 nonviral 載體的基因輸送。

### (3) 口服輸送之策略 (Delivery Strategies for Oral Route)

口服給藥是最傳統的給藥方式，藥物可以是化學性的小分子，或是大分子型的蛋白性物質。而如何有效的經由口服管道輸送蛋白性抗原體，以達到於胃腸道黏膜引發免疫作用，仍是這幾年來產學界一致努力的目標。或許這比輸送蛋白性藥物至胃腸道吸收更為可能，因為無須經由真正的吸收過程，在黏膜表面的淋巴系統或許就可扮演引發免疫作用的啟動器。但此些蛋白性抗原的輸送仍需解決眾多的問題，才能達到實用性的階段。一者需要去除抗原的可能毒性，且必須保護此些蛋白性抗原經過胃酸之侵蝕與酵素的破壞，而如何有效的達到階段性引發免疫卻是必須解決之大問題。Dr. Alving 提出結合 Liposome 與 emulsion 以作為疫苗載體，而也證明其實際的可行。利用 Lipopeptide 性疫苗以引發細胞性反應，也為 Dr. Guillet 證明可行。至於大分子的蛋白性物質之傳送，利用 Polyacrylic acid 或 Chitosan 兩者都證明是為可行的載體，經由十二指腸的給藥，可明顯增加 Insulin 的吸收而有效的控制血糖。但此些系統皆是受到處方設計及藥物本身所影響，如能有一個口服系統能降低處方設計之研究以及能適用於各種藥物之需求，將是解決口服管道吸收之基本問題。

### **(三) 攜回資料名稱及內容**

Proceedings of 27th International Symposium on Controlled Release of Bioactive Materials (2000) 為本次大會所有演講和壁報的論文集，是以 CD 儲存，每篇都有兩頁摘要，可供國內有興趣的學者專家之參考。

# CHARACTERIZATION OF COLLAGEN GEL SOLUTION AND COLLAGEN MATRIX FOR CELL CULTURE

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## Introduction

The necessity of artificial skin substitutes for wound healing or model membranes for penetration studies promotes the development of a two-layered design with a sheet of keratinocytes attached to a biocompatible collagen-based substrate. Collagen and its derived matrices have, therefore, become a popular biomaterial for this purpose and been suggested as bioprotheses for burn wound dressings<sup>1</sup>, tissue templates, etc, due to their compatibility<sup>2</sup>. Glutaraldehyde is the most widely used reagent for chemical crosslinking to minimize contraction of the collagen gel during cell growth but its use is associated with marked cytotoxicity<sup>3,4</sup>. Further incorporation of glycosaminoglycans (20% chondroitin sulfate)<sup>5</sup> or hyaluronic acid into<sup>6</sup> collagen gels, and subsequent crosslinking with carbodiimides and diamines or glutaraldehyde is able to enhance the strength of the collagen gels thus inhibiting fibroblast to contract the collagen matrices. In this study, the collagen were modified by crosslinking with various concentration of glutaraldehyde. Physical properties of these crosslinked collagen gel solutions and the resulting freeze-dried collagen matrices were characterized and evaluated for compatibility with fibroblasts.

## Experimental Methods

Telopeptide-poor collagen was harvested as previously described [3]. 1% w/w collagen gel solution was prepared by dissolving collagen in 3% acetic acid. Various concentration of glutaraldehyde was added and placed at 4°C for 24 h. After then, these gel solutions were subjected to the measurement of viscoelasticity by DMA. A portion of above gel

solution was transferred into a plastic container (3 cm in diameter) and freeze-dried to form collagen matrices with a thickness of about 0.2 to 0.3 mm. Mechanical strength of these collagen matrices were monitored by DMA.

Dynamic Mechanical Analyzer (Perkin — Elmer, DMA7e) was used to measure the viscoelasticity of gel solution by following the creep compliance curve and the mechanical strength of the matrices by monitoring the time-modulus curve. The gel solution was placed in the cup. A cup-plate testing geometry was used with a creep & recovery scan mode. After equilibrating for one minute, 2 mN force was placed on the samples for 3 minutes and returned to zero thereafter. The studied matrix was mounted on a stainless steel parallel extension kit and the measurement was conducted at ambient temperature. The initial force applied was 5 mN and the extension rate was 100 mN/min. The modulus and tensile strength for collagen matrices at break point were monitored.

Nude mouse skin samples were obtained from National Animal Center, NSC, ROC. The epidermis and dermis were separated by floating the skin on 0.5% (w/v) dispase in phosphate buffered saline (PBS) at 4 °C overnight. Fibroblasts were isolated by mincing the dermis, and these were grown in DMEM supplemented with 10% (v/v) foetal calf serum (FCS). Cells were used for experiments when they were three passages old, and the seeding density on collagen matrices was  $3 \times 10^5$  cells/ml.

## Results and Discussion

The extent of crosslinking after treating collagen gel solutions with various

dissolution of nifedipine with the presence of pluronic F-68 was gradually increased with increasing the ratio of pluronic F-68 to nifedipine. Enhancement of hydrophilicity of nifedipine with the help of pluronic F-68 was another possible reason.

Figure 1B displays the release of nifedipine from formulations with different nifedipine ratio of the inner layer to the outer layer with a fixed ratio of pluronic F-68 to nifedipine in both layers. The comparison between N1 and N2 demonstrates that the increase of the nifedipine ratio in the inner layer enhances the initial rate of drug release. The comparison between N2 and N5 illustrates that the increase of the ratio of pluronic F-68 to nifedipine in the inner layer was further able to promote the initial release of nifedipine. The latter shows a release profile close to that for Coracten.

With a fixed nifedipine ratio of 1.5 between the inner layer and the outer layer, the influence the ratio of pluronic F-68 to nifedipine in the outer layer on the initial release of nifedipine was displayed in Figure 1C. As expected, increasing this ratio increases significantly the initial release rate of nifedipine. This is due to the enhancement of hydrophilicity of nifedipine or solubility with the presence of pluronic F-68. However, with this nifedipine ratio, the initial release rate was faster but the latter release rate was slower with a comparison to that for Coracten.

By increasing the nifedipine ratio for the inner layer to the outer layer to a fixed value of 1, the thickness of controlling membrane was manipulated with the coating of different amount of Surelease. Figure 1D illustrates that the increase of coating percentage decreases the release rate of nifedipine from the inner layer. According to Fick's first law, increasing the thickness of controlling membrane decreases the concentration gradient between both sides of the controlling membrane resulting the decrease of release rate across the membrane.

In conclusion, the use of pluronic F-68 to

enhance the nifedipine dissolution was applicable to a multiple-layer design of controlled release pellets of nifedipine. Proper adjustment of the nifedipine ratio between the inner layer and the outer layer and the ratio of pluronic F-68 to nifedipine in the outer layer was able to have a release profile comparable with the necessity of clinical use.

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Table 1. Multiple-layered Design and Formulation

| Rx                  | N-1              | N-2 | N-3          | N-4 | N-5         | N-6 | N-7 | N-8 |
|---------------------|------------------|-----|--------------|-----|-------------|-----|-----|-----|
|                     | Inner Layer      |     | Middle Layer |     | Outer Layer |     |     |     |
| Drug (N)            | 1.5 <sup>a</sup> | 1.5 | 1.5          | 1.5 | 1.5         | 1.0 | 1.0 | 1.0 |
| EC (%) <sup>b</sup> | 5.0              | 5.0 | 5.0          | 5.0 | 5.0         | 5.0 | 7.5 | 10  |
|                     | Inner Layer      |     | Middle Layer |     | Outer Layer |     |     |     |
| Drug (N)            | 0.5 <sup>a</sup> | 1.0 | 1.0          | 1.0 | 1.0         | 1.0 | 1.0 | 1.0 |
| Ratio <sup>c</sup>  | 2                | 2   | 1/2          | 1/3 | 1           | 1   | 1   | 1   |

<sup>a</sup>Ratio of the nifedipine amount in the inner layer to that in the outer layer

<sup>b</sup>% of ethylcellulose relative to the nifedipine amount in the inner layer

<sup>c</sup>Ratio of nifedipine amount in the outer layer to pluronic F68

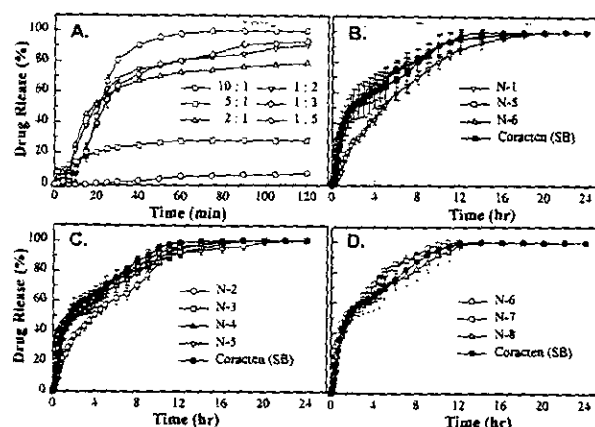


Figure 1. The release profiles of nifedipine from various formulations

# THE INFLUENCE OF CURING SPHERONIZATION ON THE CONTROLLED RELEASE CHARACTERISTICS OF PELLETS

C.-W. Lin, J.-S. Wu, L.-C. Chen, S.-C. Chen, and M.-T. Sheu

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## Introduction

Membrane controlled release of pellet dosage forms provide a better flowability, lower friability, and minimal irritation to gastrointestinal mucosa, making it becomes an interest of main application for oral controlled release dosage forms. However, the production method of pellets could affect the release pattern of drugs from pellets<sup>1,2</sup>. Preliminarily, it was found that the treatment of etofibrate powder by melting was able to improve the density of powder and the spheronization of pellets was positive to the increase of pellet density. With partial melt or softening of drug chemicals at an elevated temperature, spheronization is not only able to make pellet denser, but also to cure the controlled release membrane to an extent of as even as possible. It was thought that curing pellet with spheronization at an elevated temperature after membrane coating of pellets would have a synergetic effect on the increase of pellet density as well as the prolongation of drug release.

## Experimental Methods

### Preparation of etofibrate pellets

Etofibrate powder (200 gm) was wet massed with a water solution containing 10 gm each of PEG 6000 and acetone in a planetary mixer. The mass was immediately extruded by a cylinder type extruder with a 1.0 mm orifice screen. The resultant extrudates were placed on the cross-hatched plate of spheroniser. The spheronization speeds was fixed at 960 rpm and the residence time was 10 min. After spheronization, pellets were dried in a hot-air oven at 40°C for 10 hours.

### Film coating of pellets and curing

400 gm of etofibrate pellets were charged into the chamber of a fluidized-bed coater (Model GPCG-1). A film dispersion was prepared by mixing Surelease latex with Talc of 50% w/w of ethylcellulose and then adjusted the final solid content to 80% w/w. The dispersion of film polymer was bottom-sprayed onto the fluidized etofibrate pellets. The spraying was continuing until the designed amount of film polymer (2.5, 5.0, and 7.5% of pellet weight) was sprayed on. Finally, the film coated pellets were dried in a hot-air oven for another 10 hours at 40°C after coating.

Four jacket temperatures (ambient, 40, 45, and 47.5 °C) were chosen to examine the influence of curing spheronization on the properties of pellet. When the designated jacket temperature was reached, the film-coated pellets of etofibrate were subjected to spheronization at different conditions, three rotation speeds and two residence times.

### Dissolution studies

The USP XXI dissolution apparatus II was used to measure the release rate of etofibrate at 100 rpm. The temperature was maintained at  $37 \pm 0.5$  °C. Simulated gastric fluid containing 0.1% w/v polysorbate 80 without enzyme (pH 1.2) was used as dissolution medium. Five ml were withdrawn at predetermined time intervals and replaced with the same volume of fresh medium. Etofibrate content was determined using a UV/VIS method at the wavelength of 263 nm. Each data point is the mean of three individual trials.

concentration of glutaraldehyde was examined. It demonstrated that the extent of crosslinking of terminal amino group on collagen increased with increasing the concentration of glutaraldehyde. It approaches complete crosslinking when the used concentration of glutaraldehyde was higher than 0.12% at an 1.0% w/w concentration of collagen.

The viscoelasticity of these collagen gel solutions was measured by following the creep compliance curve. The curvature portion of all creep compliance plots can be described by a Voigt-Kelvin model: Compliance = Strain( $\epsilon$ ) / Stress ( $\delta$ ) =  $[1/\text{Young's Modulus}(E)] * (1 - \exp(-t/\tau))$ , in which  $\tau$  is designated as  $\eta/E$ . Nonlinear regressing the compliance with respective to time  $t$ , Young's modulus ( $E$ ) and retardation time ( $\tau$ ) were calculated from the corresponding equations. Results indicate that both elastic modulus and viscosity of collagen gel solution increase with increasing the concentration of glutaraldehyde. A significant increase in viscosity was noticed when the concentration of glutaraldehyde used was higher than 0.12%. Results illustrate that retardation time increases with increasing the concentration of glutaraldehyde, indicating a gradual transformation of liquid characters of collagen gel solution into solid characters as the extent of crosslinking by glutaraldehyde increases.

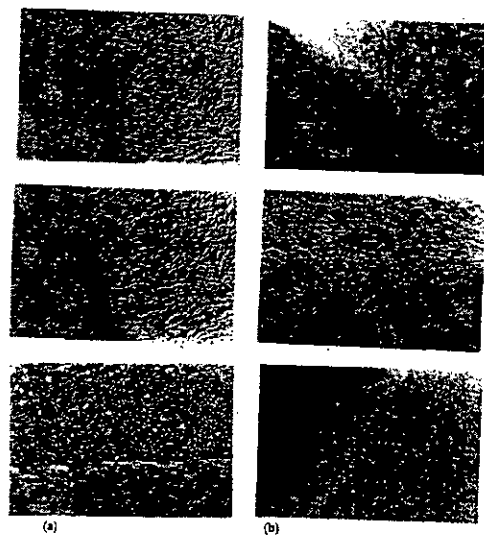
The break modulus of collagen matrices crosslinked with various concentration of glutaraldehyde was monitored by DMA at break point. It clearly illustrates that break modulus of collagen matrices increases with increasing the concentration of glutaraldehyde. A higher break modulus means a higher stress is needed to achieve a constant strain, thus indicating a higher resistance of collagen matrices to deformation with increasing the extent of crosslinking. Further, the break modulus of these collagen matrices becomes higher when the matrix was incubated with FCM before measurement. This is probably a result of enforcement of mechanical strength by

increasing hydrogen bonding between collagen fibril.

Figure 1 shows the growth of fibroblasts within the collagen matrix crosslinked with various concentration of glutaraldehyde. The compatibility of collagen matrix with fibroblasts was demonstrated. There shows no sign of cytotoxicity for fibroblasts, even within a collagen matrix crosslinked with a concentration of glutaraldehyde as high as 0.2%. The contraction of collagen matrices by fibroblasts was minimal as well.

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**Figure 1.** The growth of fibroblasts within collagen matrix

# THE INFLUENCE OF CURING SPHERONIZATION ON THE CONTROLLED RELEASE CHARACTERISTICS OF PELLETS

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## Results and Discussion

Figure 1 shows the release profiles of etofibrate from pellets with no coating. Subjecting to a curing spheronization at 40 °C, the change of spheronization speed and residence time did not modify the release rate of etofibrate in a significant extent. However, drug released from these pellets curing spheronization at 45 °C was slower when the speed of spheronization was faster and the residence time was longer.

At a 2.5% level of coating, subjecting these pellets to curing spheronization was able to improve the density at all elevated temperature examined and the speed and residence time of spheronization. Figure 2 illustrates the release profiles of etofibrate from pellets coated with a 2.5% level of membrane and processed with varying conditions. It demonstrates that at a lower temperature of curing spheronization, the increase of spheronization speed results in the faster release of drug. It is probably due to the destruction of membrane integrity at a higher spheronization speed. On the contrary, the release pattern of etofibrate from pellets subjecting curing spheronization at a higher temperature is not much different from that of pellets with no coating. The denser structure on pellet surface making the release of etofibrate slower might be the reason for that.

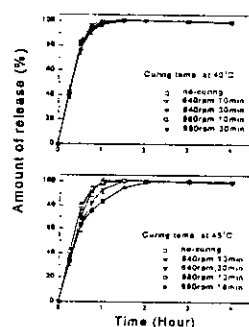
Increasing the coating amount to a level of 5.0 or 7.5% of pellet weight, the influence of curing spheronization on the characteristics of pellets was similar as above. Referring to Figure 3, pellets with a coating level of 5% provide a prolonged release of 10 hours. After curing spheronization, the release rate of etofibrate becomes faster as a result of the destruction of membrane integrity. However, the densification of local surface of pellet by subjecting to curing

spheronization at 47.5 °C makes this phenomena least obvious (Figure 4).

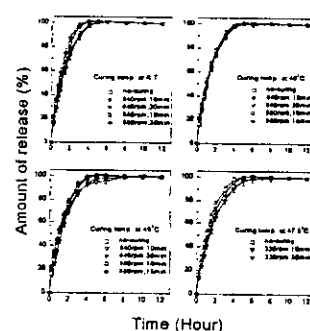
In conclusion, curing spheronization is able to improve the pellet density depending on the temperature and residence time of spheronization. Densification on the local surface of pellets might play an important role in the increase of pellet density. The prolongation of drug release from these pellets was not due to the increasing extent of membrane integrity, but as a result of denser structure of pellet surface.

## References

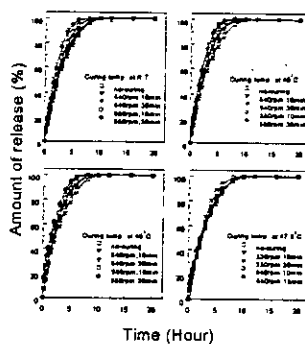
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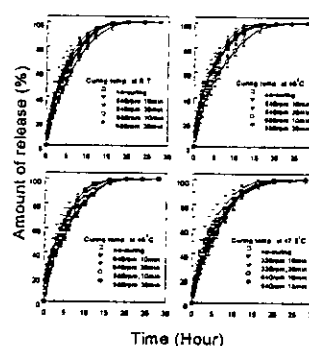
**Figure 1.** The release profiles of etofibrate from pellets with no coating.



**Figure 2.** The release profiles of etofibrate from pellets coated with 2.5%



**Figure 3.** The release profiles of etofibrate from pellets coated with 5.0%



**Figure 4.** The release profiles of etofibrate from pellets coated with 7.5%