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Interactions between collagen and perfluorocarbon emulsions

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Summary

The interactions between perfluorocarbon emulsion and both dissolved collagen and suspensions of collagen fibrils were studied in a new formulation suggested for enhancement of wound healing Since the emulsion droplets are covered with a charged phospholipid monolayer, both electrical and hydrophobic interactions may exist. Electrophoretic mobility measurements which are conducted at various pH values, and light microscopy, revealed that the emulsion droplets were adsorbed onto the collagen fibrils, thus forming a new population of particles. This phenomenon was also observed at the pH values of the isoelectric points of collagen or lecithin, indicating possible hydrophobic interactions between collagen and lecithin From adsorption experiments, the area of collagen molecule at the interface was calculated. This suggested formation of an end-on oriented collagen molecules layered at the water-perfluorocarbon/lecithin interface.

Introduction

Collagen is the most abundant protein in mammals, including man, which provides the skeletal framework, and is involved in many physiological processes of biological adaptation and tissue regeneration. Its adsorption onto various surfaces is of great importance, such as in implanted devices in which bound collagen molecules enhace adhesion of epidermal cells to the polymer surface and prevent implant failure (Von Recum and Park, 1981; Yaffe et al., 1982; Von Recum, 1984). Collagen plays an important role in the wound healing process, in which it initiates the platelet release reaction, enhances the migration of fibroblasts to the site of injury and forms the scar tissue (Shoshan, 1981). Perfluorocarbon (PFC) emulsions have been investigated as blood substitutes owing to their high oxygen and CO_2 solubility in the emulsion droplets (Yokoyama et al., 1978).

There are many instances in which wounds evolve or fail to heal because of inadequate oxygen supply to the tissue (Silver, 1984). Thus, it has been assumed that supplementation of collagen with PFC would enrich the wound environment with the necessary oxygen. Indeed, animal studies have shown that a preparation of collagen and PFC emulsion enhanced healing of excised skin wounds (Shoshan et al., 1990). The PFC emulsion is stabilized by a monolayer of phospho-

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lipids (Magdassi and Simon-tov, 1990) and, therefore, both electrostatic and hydrophobic interactions between the collagen and the emulsion droplets can take place.

The purpose of this study was to investigate the interactions between the perfluorocarbon emulsion droplets with both collagen molecules and collagen fibrils.

Materials and Methods

Perfluorodecalin (Aldrich) and the surfactant Lipoid 80 (Lipoid KG, Germany) were used without further purification. According to the manufacturer, Lipoid 80 is a purified egg lecithin which contains 80–83% w/w phosphatidylcholine, 8–11% w/w phosphatidylethanolamine, 2–3% w/w sphingomyelin, max. 3% w/w lysophosphatidylcholine, and 1% w/w cholesterol. The content of choline is 13–14 wt%, and that of phosphorus is 3.5–3.8% w/w.

Acid-soluble and tritium-labeled collagen was extracted from guinea pig skin and purified according to the method described by Shoshan et al. (1967).

The emulsions were prepared as follows:

A concentrated solution of lecithin (10% w/w) in distilled water was obtained by sonication for 10 min, at 5°C. This concentrated solution was further diluted with water to yield a final concentration of 2% w/w, and was homogenized for 2 min. To this solution the perfluorodecalin was added (10% w/w) and the mixture was homogenized for 5 min using a Silverson homogenizer.

The droplet size distribution of each emulsion was measured on a Coulter counter, model TA II (Coulter Electronics, Ltd.).

Electrophoretic mobility (EPM) measurements of emulsions without collagen were performed by diluting 0.25 ml of the original PFC emulsion, in 40 ml NaCl solutions of varying concentrations. These were adjusted to the required final pH by HCl or NaOH, and yielded a final ionic strength of 0.01. In order to maintain a low ionic strength, no buffer salts were used. EPM measurements of the collagen fibrils were performed by diluting 1 ml collagen solution (2.5 mg/ml, 0.01 M HCl) in 40 ml NaCl solution containing varying concentrations of the salt. The required pH was adjusted by NaOH, and the final ionic strength was also 0.01.

EPM of the mixture of PFC emulsion and collagen was measured after mixing (100 min) 0.25 ml PFC emulsion with 39.75 ml of the dispersed collagen fibrils. Each EPM measurement was performed by measuring the electrophoretic mobility of at least 150 particles, at 20°C using a Zeta meter (Zeta Meter, Inc.).

The adsorption experiments were performed as follows: 1 ml of ^{3}H-labeled collagen solution (phosphate buffer, pH 7.6, I 0.4) was mixed with 0.25 ml PFC emulsion, for 24 h, at 5°C. The PFC emulsion droplets were than separated from the aqueous phase by centrifugation (10 min, 6200 × g). No collagen is sedimented under such centrifugation conditions. The upper transparent liquid phase, which was free of emulsion droplets, was analyzed by liquid scintillation counting (Tricarb 460C, Hewlett Packard) and the concentration of the radioactive collagen in solution was determined. The adsorbed collagen was calculated from the difference in the dpm counts of the original collagen solution and that of the separated upper aqueous phase after adsorption.

The adsorption experiments were performed at collagen concentrations of 0.1–3.5 mg/ml. The collagen solution containing 3.5 mg/ml was taken as the highest possible concentration. The concentration of these solutions was determined based on the hydroxyproline content (Bergman and Loxley, 1963). Since the hydroxyproline content of collagen preparation varies between 10 and 14% w/w, it was more accurate to measure hydroxyproline concentration as an expression of collagen concentrations in the adsorption experiment.

Results and Discussion

Both the emulsion droplet and collagen fibrils are electrically charged. The emulsion droplets possess a negative charge above pH 3 (Sabet et al., 1982; Magdassi and Siman-tov, 1990) due to the adsorbed phospholipids at the oil-water inter-

face, and the collagen is positively charged below its isoelectric point, pH 9.4 (Eastoe, 1967). Therefore, it could be expected that measurement of the electrophoretic mobility would give an indication of possible interactions between the two components.

Fig. 1 shows EPM of PFC emulsion, of collagen fibrils, and of the two components mixed together.

Fig. 1a clearly indicates that the emulsion droplets are negatively charged (pH 6.5, $I = 0.01$), and Fig. 1b shows that the collagen fibrils are positively charged, at the same pH and ionic strength. However, When the emulsion was mixed with the collagen dispersion, a new pattern of EPM was obtained (Fig. 1c), in which the particles were still negatively charged, but the EPM shifted to a position between that of either of the components of the mixture.

Only a single histogram of particles' population vs EPM was obtained for the mixture, indicating the formation of a new single population of particles, as verified by a statistical t-test. Therefore, the particles present in the combined system are either emulsion droplets adsorbed onto the collagen fibrils, or collagen fibrils adsorbed onto the emulsion droplet. Fig. 2 shows the light microscopic appearance of collagen fibrils formed from the solution of 0.625 mg/ml at ionic strength 0.01 and pH 6.5 with PFC emulsion droplets, adsorbed onto them.

Since the pH in which the above experiments were conducted was 6.5, i.e., the emulsion droplets are negatively charged whereas the collagen fibrils are positively charged, it is likely that the interaction between the collagen and the emulsion is mostly electrostatic. In order to evaluate whether only electrostatic interactions exist in the combined system, similar experiments were performed at pH values varying from the isoelectric point of lecithin (pH 3), up to the isoelectric point of collagen (pH 9). Fig. 3 shows average EPM values calculated for each pH value. It appears that here too the values of the EPM of the particles in the mixture are between the separately measured EPM values of the PFC droplets and the collagen fibrils. The new point of zero charge for the mixture was obtained at pH ~ 5. It

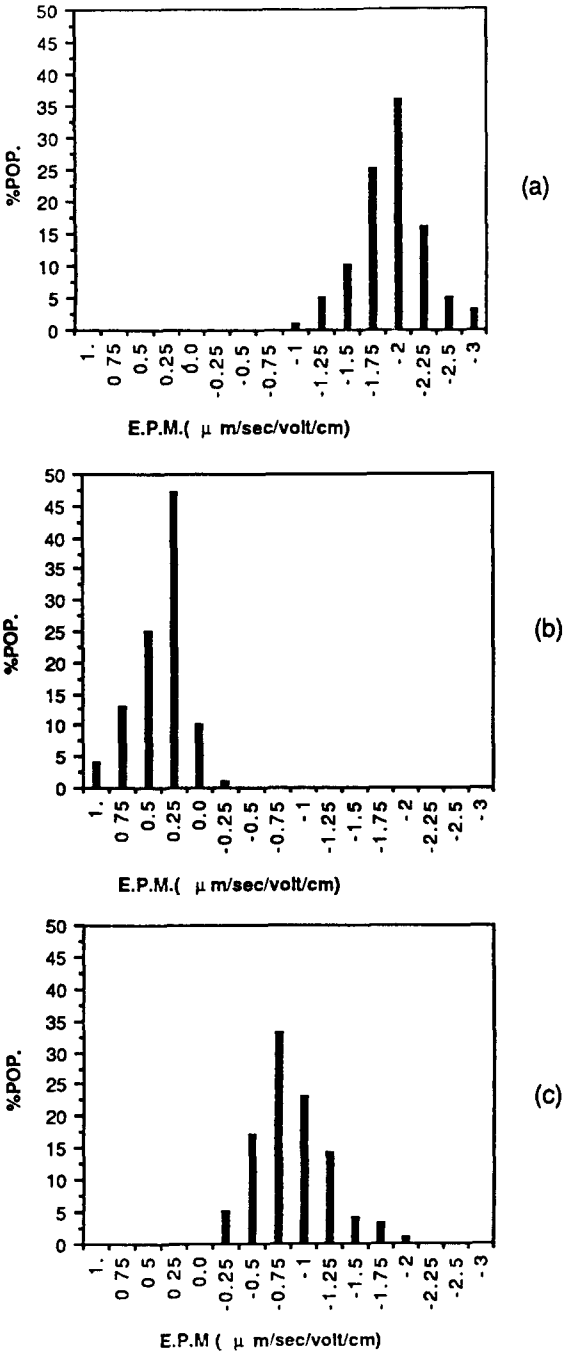

Fig. 1 Electrophoretic mobility (EPM) of PFC emulsion (a), collagen fibrils (b) and a mixture of PFC emulsion and collagen fibrils (c). All measurements were performed at ionic strength I 0.01, pH 6 5 (for details see text)

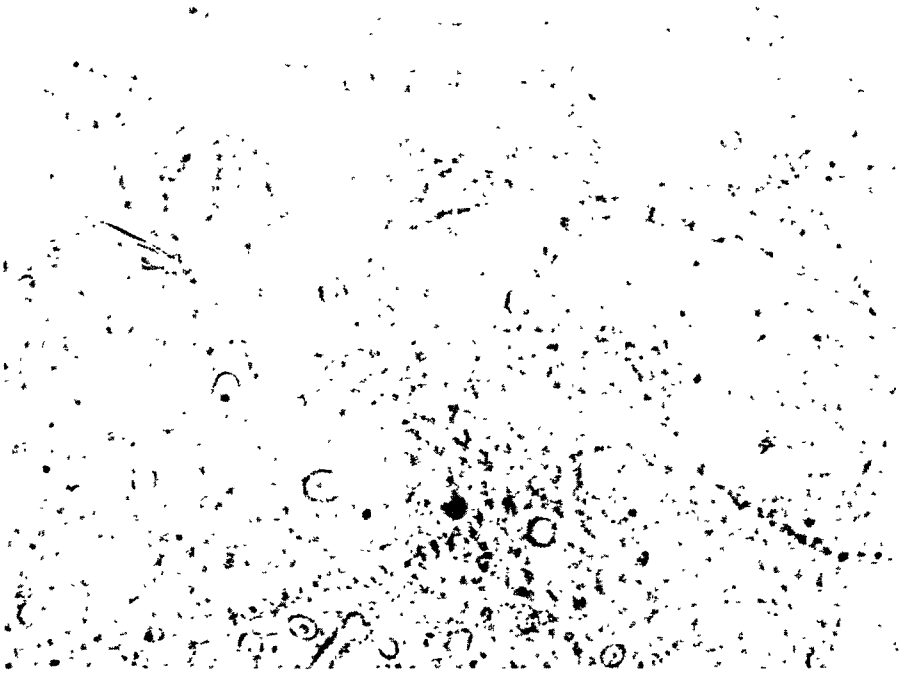

Fig 2. Light microscopy of a mixture of PFC emulsion and collagen fibrils (I 0 01, pH 6 5)

is of interest to note that at the pH which corresponds to the isoelectric point of either collagen or lecithin, only one population of particles was observed. Therefore, since at these pH values only one of the components (lecithin or collagen) is charged, it should be concluded that non-electrostatic interactions may also contribute to the overall interaction between collagen and the emulsion droplets. This conclusion is in agreement with the data obtained by surface tension measurements (Bagnall, 1978), indicating hydrophobic interactions between the hydrophobic residues of the collagen molecules and various oil phases.

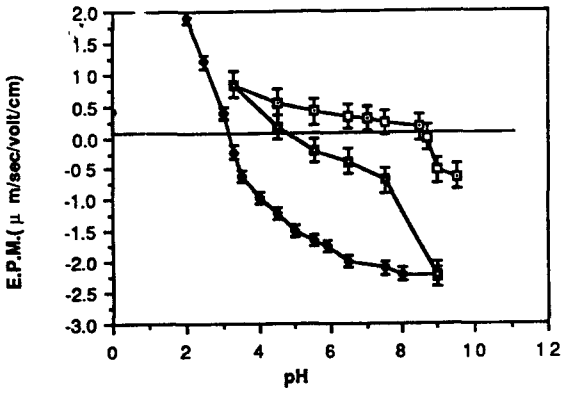

Fig 3 Average EPM of PFC emulsions (♦), collagen fibrils (□), and a mixture of emulsion droplets and collagen fibrils (□), at various pH and constant ionic strength (I 0 01).

The described experiments on collagen fibril-PFC interactions were carried out at low ionic strength. Under such conditions, collagen molecules in solution form a suspension of fibrils and the results so far were related to emulsion-fibril interactions only. At high ionic strength ($I = 0.4$), however, the collagen molecules remain in solution. Therefore, at such ionic strength the adsorption of collagen molecules can be measured directly in solution using a ^{3}H-labeled collagen. The adsorption experiments were conducted at pH 7.6 and I 0.4, i.e., under conditions in which the collagen molecules are positively charged, and the electrical interactions should be insignificant, due to compression of the electrical double layer.

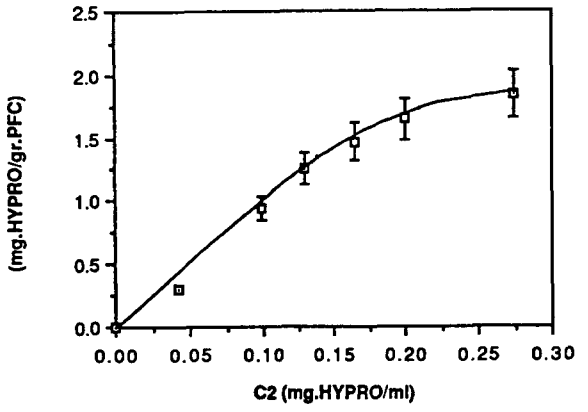

Fig 4 Adsorption isotherm of collagen onto PFC emulsion droplets The concentration is expressed by the concentration of hydroxyproline (HYPRO). C_2 denotes the concentration in solution after adsorption

As can be seen from the adsorption isotherm (Fig. 4), the adsorbed amount of collagen increased, as expected, with the increase in collagen concentration in solution. However, although the highest possible initial concentration was used, no plateau was observed. The highest adsorbed amount was 1.84 mg hydroxyproline/mg PFC, which is equivalent to 13.1–18.4 mg collagen/mg PFC. From this concentration, and knowing the average droplet diameter (9.8 μm), it appears that the surface concentration of collagen is very high, namely 42–60 mg/m^2. For comparison, the adsorption of collagen reported for various surfaces was between 1 and 25 mg/m^2 (Penners et al., 1981; Silberberg and Klein, 1981; Deyme et al., 1986). The high degree of adsorption could result either from multilayer adsorption, or from a closely packed monolayer, in which the collagen molecules are oriented in an end-on configuration. Using the highest adsorption concentration, it appears that the area occupied by a collagen molecule is 830–1180 Å^2, which is larger than the smallest possible area for a collagen molecule (175 Å^2), when oriented perpendicularly to the surface (Deyme et al., 1986). From adsorption experiments performed for surfaces such as glass, teflon and silconized glass, it was concluded that the collagen molecules are indeed adsorbed like brush bristles rooted perpendicularly to the surface (Penners et al., 1981).

It was shown that when functional hydrophilic groups were formed at the surface of a hydrophobic polymer and were oriented perpendicularly to the polymer surface, the protein adsorption was enhanced (Proust et al., 1983; Bazkin et al., 1984). In the present study, the PFC emulsion droplets present a hydrophobic surface (PFC and hydrophobic chains of the lecithin), and anionic groups which are oriented towards the aquéous phase. Thus, a similar perpendicular orientation of the protein, i.e. collagen, occurs upon adsorption onto PFC emulsion droplets. Moreover, the high adsorbed collagen concentration apparently results from the favorable adsorption conditions, which allow both electrical and hydrophobic attraction, and concomitant suppression of the repulsion between neighboring collagen molecules.

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