
Collagen at interfaces I. *In situ* collagen adsorption at solution/air and solution/polymer interfaces

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Collagen was isolated from rat tail tendons and acetylated with ^{14}C acetic anhydride. *In situ* adsorption of this collagen from a buffer solution (pH = 2.7) was measured at the interfaces to air, polyethylene and polyethylene grafted with poly(maleic acid), respectively.

The kinetics of adsorption were recorded for all surfaces studied and the corresponding diffusion coefficients for collagen in solution with various protein concentrations were calculated. The desorption

of collagen from polymer surfaces was also studied.

These experiments reveal the existence of both a reversibly and an irreversibly adsorbed collagen layer on the polymers tested. The desorption/adsorption ratio for the polyethylene is higher than that for the grafted polyethylene indicating stronger interactions of collagen with the grafted surface than with the non-modified polyethylene.

INTRODUCTION

Collagen is the most abundant protein in the animal kingdom, including man. It is a major component of the extra-cellular matrix in many tissues, and its metabolism is directly associated with many physiological processes of biological adaptations and tissue regeneration. Many vital processes of tissue repair and growth following injury of any kind depend largely on the rate of self-assembly of collagen molecules.¹⁻³

The application of collagen for the manufacturing of different dermal collagen-based preparations used as subcutaneous implants and as replacement for dermis in skin wounds was recently explored.⁴⁻⁶

Also the development of a suitable percutaneous device that would satisfy current implant requirements is directly related to the histological changes surrounding a percutaneous implant. Collagen molecules bound to the implanted material enhance adhesion of epidermal cells to the polymer surface and prevent implant failure.⁷⁻⁹

A sound understanding of the factors influencing collagen interactions with solid surfaces is therefore of great importance in medicine and pharmacology.

Our aim was to investigate the behavior of collagen molecules at different interfaces by studying the *in situ* adsorption of this protein alone and in

competition with another protein. The results of this investigation are presented in separate articles.

MATERIALS AND METHODS

Extraction and isolation of collagen

Collagen was isolated from rat tail tendons using a procedure outlined by Chandrakasan et al.¹⁰ The lyophilized collagen was stored at -20°C and dissolved before use in a (0.2M NaCl – 0.1M CH_3COOH adjusted to $\text{pH} = 2.75$ with concentrated HCl) buffer solution (acetic buffer). Acidic pH value was chosen in order to minimize the association of collagen molecules in solution.

Preparation of ^{14}C acetylated collagen solutions

Acetylation of collagen was carried out according to the method previously used for ^{14}C labelling of bovine submaxillary mucin.¹¹

Lyophilized collagen (57 mg) was dissolved in 6 mL of dimethyl sulfoxide (DMSO) at room temperature. Gentle stirring during 24 hrs ensured complete dissolution of the collagen. The solution was then centrifuged for 20 min. To this solution 0.5 μl of [$1\text{-}^{14}\text{C}$] acetic anhydride was then added. After 3 hrs of gentle stirring, 6 mL of distilled water was poured into the solution and the whole mixture was dialysed against seven changes (1 liter each) of distilled water. The collagen concentration in the solution after dialysis was 0.59 mg/mL as measured by the Lowry method.¹² For adsorption experiments this solution was diluted with the acetic buffer to give solutions in the $2 \times 10^{-3} - 5 \times 10^{-2}$ mg/mL concentration range.

The percentage of NH_2 -groups acetylated during the labelling reaction was found to be about 80% as determined by the colorimetric ninhydrin-reaction according to Yemm and Cooking.¹³

Measurement of ^{14}C labelled collagen specific activity

To obtain the specific activity of the ^{14}C labelled collagen, a reference ^{14}C labelled compound, of a known specific activity (^{14}C hexadecyltrimethylammonium bromide) was used. A known amount of ^{14}C hexadecyltrimethyl ammonium bromide was deposited on a plane glass surface and evaporated. When dried its radioactivity was measured and compared with the radioactivity of the known amount of ^{14}C labelled collagen, deposited in the same manner on a glass surface, and counted in the same geometrical conditions as used for the reference.

Radioactive materials and reagents.

- (1) Acetic anhydride 1-¹⁴C with an acetic acid content [1-¹⁴C] not greater than 12% and specific activity 1.07 mCi/mg, obtained from Amersham, (Amersham, U. K.) was used for the collagen labelling.
- (2) Hexadecyltrimethylammonium bromide ¹⁴C from Amersham (Amersham, U. K.) with the specific activity 14.9 μ Ci/mg was used to estimate the specific activity of labelled collagen ¹⁴C.
- (3) Methyl methacrylate ¹⁴C from Amersham (Amersham, U. K.) was used as a reference solid source to determine the absorption of radiation by polymer samples (see adsorption measuring techniques).
- (4) Potassium thiocyanate ¹⁴C from Amersham (Amersham, U. K.) with the specific activity 58 mCi/mmole was used to measure the contribution of the solution to the radioactivity measured during collagen ¹⁴C adsorption experiments (see adsorption measuring techniques).
- (5) All non-radioactive reagents used were from Merck (Darmstadt, FRG), analytical grade. Water was tridistilled from a permanganate solution using Pyrex apparatus.

Preparation of polymer samples for adsorption measurements

The low density polyethylene film (Cryovac L film, manufactured by Grace, France, thickness 19 μ m, density 0.929 g/cm³) was cut with a circular punch (30 mm in diameter). The obtained polymer samples were washed with carbon tetrachloride and then extracted with boiling acetone for 5 hrs. After extraction the samples were dried under reduced pressure at room temperature and used for the adsorption experiments.

Grafting of polyethylene films was achieved through the free radical polymerization of maleic anhydride in acetic anhydride and in the presence of benzoyl peroxide as initiator. Purified polyethylene specimens were pre-heated at 90°C for 1 h in an oven to ensure their dimensional stability during the grafting reaction. Then they were placed in glass reaction tubes with ground glass stoppers which contained 15 mL of 30% solution of maleic anhydride in acetic anhydride and 0.2% w/w of benzoyl peroxide. The polymerization was carried out at 90°C and the reaction was stopped after 6 hrs. Grafted samples were then thoroughly extracted with boiling acetone during 5 hrs and hydrolyzed in water at 90°C. After their hydrolysis they were dried under reduced pressure at room temperature.

This employed grafting procedure binds functional groups essentially at the surface of the polyethylene carrier. Surface properties of these grafted films were characterized in our previous studies.^{14,15}

Adsorption measurement techniques

The principle of the method is based upon the use of ^{14}C labelled adsorbing substances. ^{14}C emits β -soft radiation which has a mean free path of $160\ \mu\text{m}$ in aqueous solution. This means that all radiation originating from the solution below this depth is attenuated. The measured radioactivity above the solution interface corresponds to the molecules adsorbed in excess at the interface plus that of a thin layer of solution (about $160\ \mu\text{m}$).

Two distinct techniques were used to measure *in situ* adsorption of collagen at the interfaces. They are illustrated in Figure 1a-b.

To measure adsorption at the solution/air interface we use the device schematically represented in Figure 1a. A circular glass container (1.8 cm in diameter, volume about 3 mL) is filled with ^{14}C collagen solution. Its radioactivity is measured on a recorder as a function of time up to 20 hrs. The total measured radioactivity (A_t) originated from the collagen adsorbed in excess at the solution/air interface (A_{ad}) and from that of the collagen molecules in solution close to the interface (A_s). The A_s value is proportional to the collagen concentration in solution and is determined with the help of a separately conducted experiment in which instead of ^{14}C collagen a non-adsorbing substance containing the same radioactive element (^{14}C -potassium thiocyanate solutions) was used. Then the A_s radioactivity is given by:

$$A_s = A'_s \frac{Cp}{C'p'}$$

where C and C' are the concentrations of surface active and surface non-active solutions; p and p' their respective specific activities.

Subtraction of A_s from A_t gives A_{ad} for each of the studied collagen concentrations in solution.

To measure the *in situ* adsorption on polymers, the device represented in Figure 2b was used. A specially constructed circular glass container with

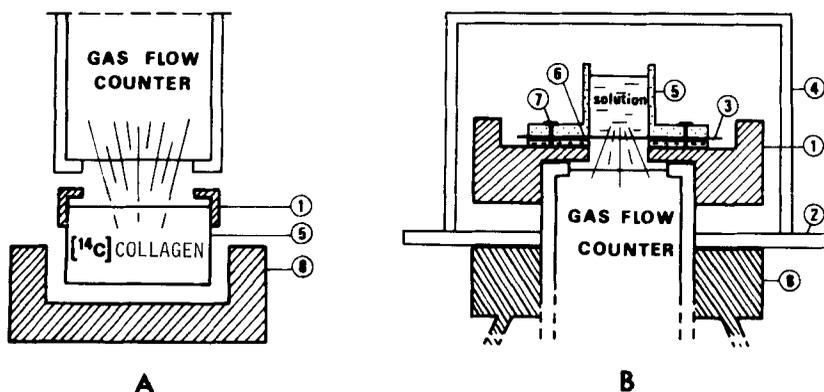


Figure 1. Adsorption measuring devices (a) for adsorption at solution/air interface; (b) for adsorption on polymer surfaces. (1), (2), (8) supports ensuring reproducibility of geometrical conditions; (3) polymer sample; (4) cover; (5) glass cell; (6) "O" ring; (7) cell assembling screws.

assembling screws enables to form a cell with a polymer window at the bottom. Molten paraffin is spread onto the flat ground part of the glass cell and the glass container is tightly sealed by means of Viton "O" rings. The cell is filled with ^{14}C collagen solution (about 3 mL) and placed in a special support above the gasflow counter. To measure the A_s value the same procedure with $^{14}\text{CNS}^-$ ions, as above, is used. In addition, the A_t value has to allow for the absorption of radiation by the polymer window. The magnitude of this correction for each polymer sample was determined with the help of ^{14}C methyl methacrylate solid source placed above the polymer window and in the same geometrical conditions as for the adsorption measurements.

For both adsorption techniques used, a calibration graph is needed to convert the adsorption values from counts/min into $\mu\text{g}/\text{cm}^2$. Details of the method, used for the first time for adsorption measurements of bovine sub-maxillary mucin, may be found in.^{11,16}

To ascertain that no preferential adsorption of the labelled collagen occurred on the studied surfaces a necessary check has been performed. The adsorbed quantities have been measured from the collagen solutions having a constant total collagen concentration (10^{-2} mg/mL) and variable ratio (ranging from 20 to 100%) of the labelled to the total collagen concentration. The amount of adsorbed collagen was independent of the labelled/unlabelled ratio in the mixture.

Desorption experiments

The *in situ* desorption experiments on polymer samples were performed after 20 hours of adsorption. Without touching the adsorption cell, which remained in its initial position, the collagen solution was pumped out from the cell with a syringe and was simultaneously replaced by the acetic buffer. Multiple replacement operations led to a negligible collagen concentration in the cell. The decrease of the radioactivity was monitored directly on a recorder and the constant values after desorption of the loosely held collagen molecules were obtained after about 15 minutes. This radioactivity corresponded to the irreversibly adsorbed collagen molecules on polymer surfaces under the conditions of the described desorption procedure. The measured radioactivity after allowing for the absorption of radiation by a polymer film, was directly converted into the surface concentration of the irreversibly adsorbed collagen (the A_s value was zero in all desorption experiments).

Subtraction of the irreversible adsorption from the total adsorption yields for each experiment the reversible adsorption (= loosely bound collagen fraction of the total adsorbed layer).

RESULTS

Figure 2 shows the time course of collagen adsorption at the solution/air interface for three different concentrations of collagen in solution. Adsorption during the first few minutes was very rapid, followed by a slower

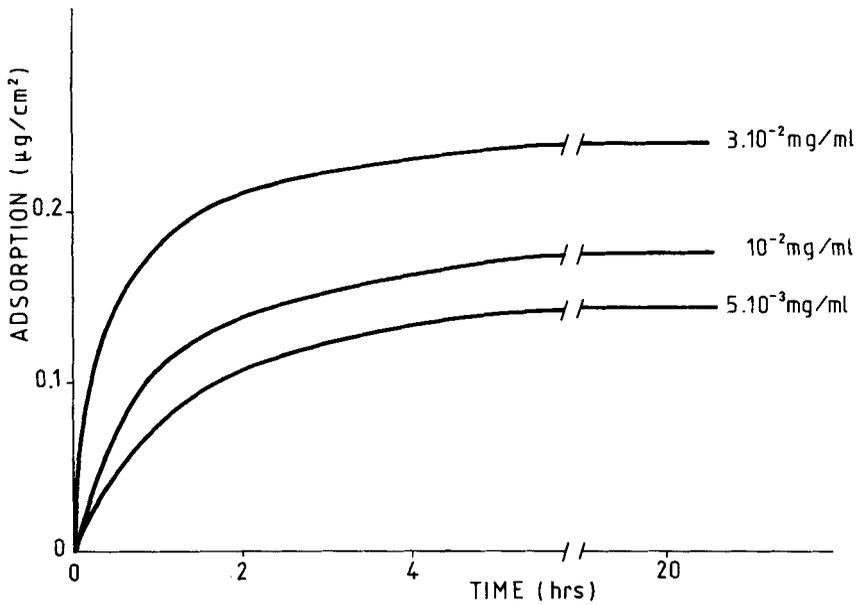


Figure 2. The time course for the collagen adsorption at the solution/air interface.

and more gradual increase. The adsorption saturation seems to be achieved after 5 hrs.

Figures 3 and 4 show the surface concentration-time curves for various solution concentrations on polyethylene and poly(maleic acid) grafted polyethylene. It seems that for both systems and at low collagen concentration

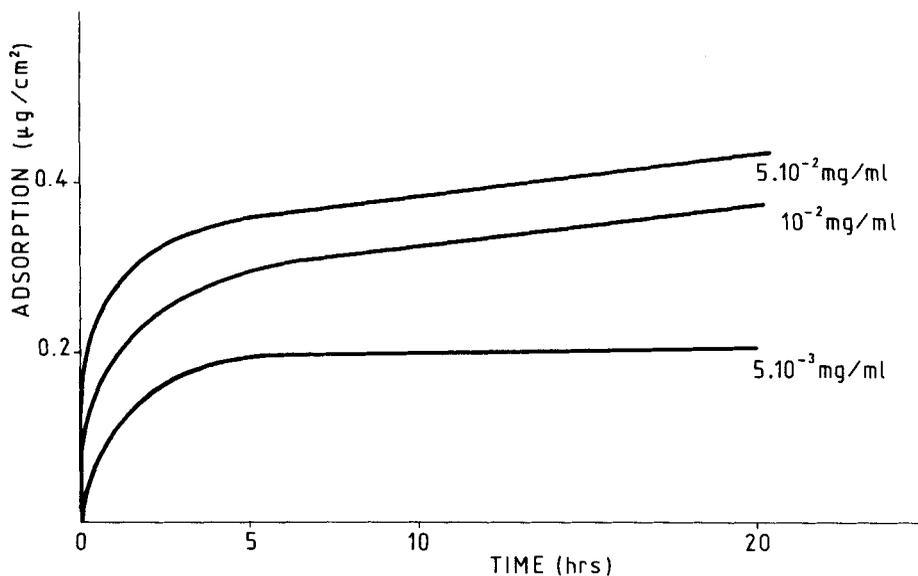


Figure 3. The time course for the collagen adsorption to polyethylene film.

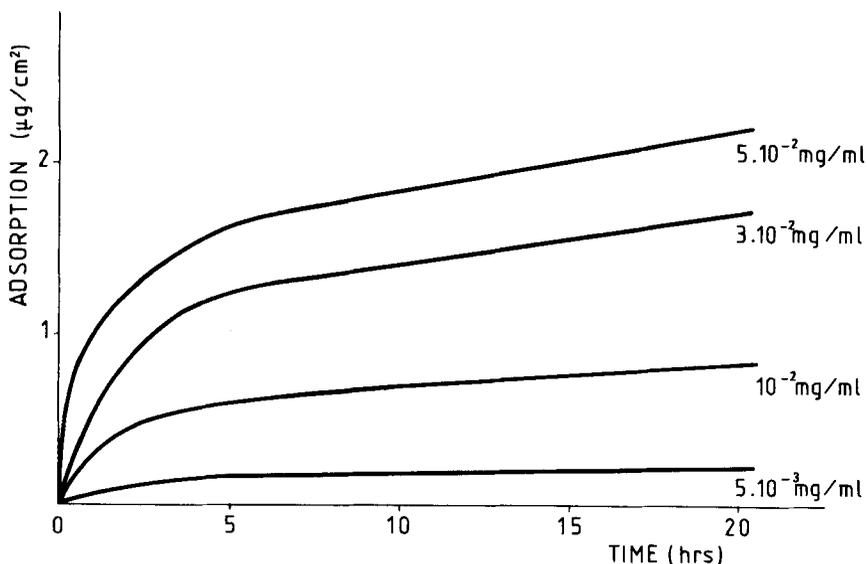


Figure 4. The time course for the collagen adsorption to poly(maleic acid) grafted polyethylene film.

in solution (5.10^{-3} M), a plateau value of adsorption is achieved after about 7 hrs. For higher collagen concentrations in solution, however, the surface concentration of collagen constantly increases with time.

Figure 5 gives the 5 h isotherms of collagen adsorption on the three different interfaces. It can be seen that at the solution/air and at the solution/polyethylene interfaces adsorption reaches a plateau value at the 0.03 mg/mL collagen solution concentration. Very high and concentration dependent adsorption can be observed on grafted surfaces. For these surfaces no adsorption plateau value is present up to the 0.05 mg/mL collagen solution concentration.

When the amounts of desorbed (reversibly adsorbed) collagen are plotted against the adsorbed quantities after 20 hrs (Figure 6), the desorption/adsorption ratio for the lower parts of the curves is 0.2 for the polyethylene and 0.05 for the grafted polyethylene. For both surfaces a sharp increase in the desorption-adsorption curves can be observed, the slopes of these curves becoming equal to one. The abissa values at the break-up points of the desorption-adsorption curves (0.3 and $0.37 \mu\text{g}/\text{cm}^2$) would correspond to the maximum amount of irreversibly adsorbed collagen on the polyethylene and the grafted polyethylene surfaces, respectively.

DISCUSSION

The collagen molecule is composed of three continuous helical polypeptide chains, each having a molecular weight of about 10^5 and containing about 1000 amino acid residues. The presence of considerable amounts of hydroxyproline and hydroxylysine favor an interwinded triple helix stiff structure.³

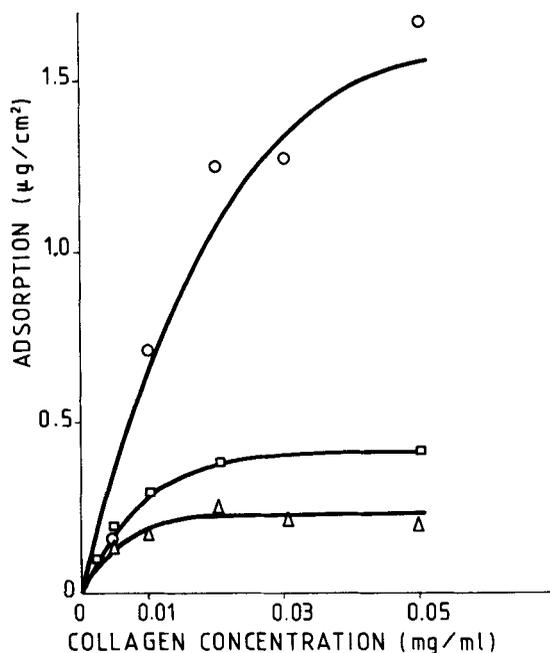


Figure 5. Adsorption of collagen to various surfaces after 5 hrs. Δ air; \square polyethylene; \circ poly(maleic acid) grafted polyethylene.

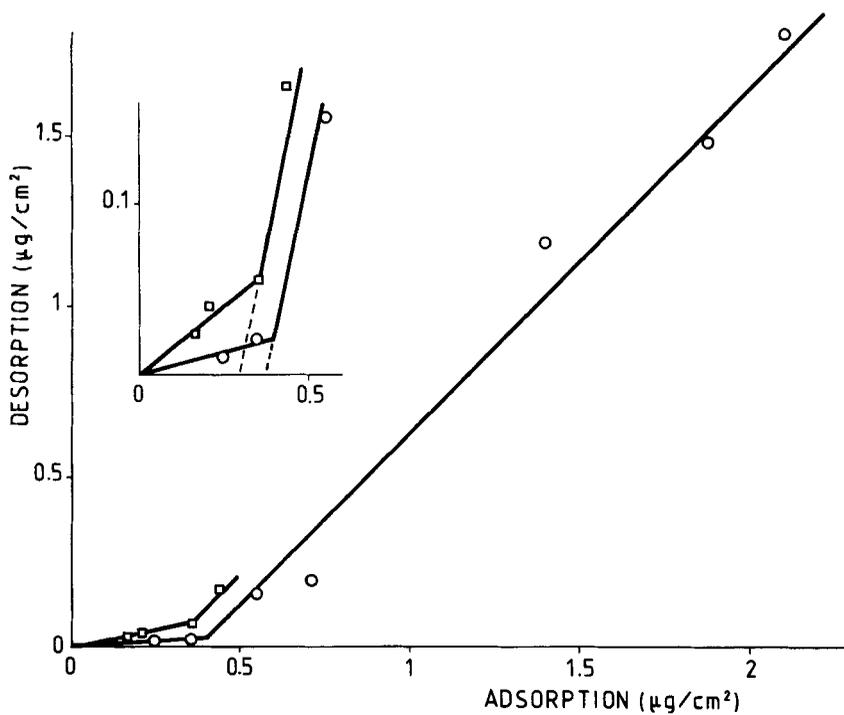


Figure 6. Desorption/adsorption relationship. \square polyethylene; \circ poly(maleic acid) grafted polyethylene. The initial parts of both curves with the abscissa values of the break-up points are shown in the enlarged inset.

The molecule can thus be regarded as a rigid rod, about 3000 Å long and 15 Å in diameter.² If these molecules are lying flat in a "side-on" position then a close-packed layer will accommodate $2 \cdot 10^{11}$ molec/cm² or 0.1 μg/cm². In an "end-on" close-packed position $5 \cdot 10^{13}$ molecules will occupy 1 cm² which represents 25 μg/cm².

The adsorbed quantities on all studied surfaces were much higher than the 0.1 μg/cm² which would only correspond to a flattened monolayer described above. The possible configurations are therefore as follows: (1) "side-on" lying collagen molecules built up to multi-layers; (2) the collagen rods standing perpendicularly to the surface in an array like brush bristles ("end-on" position); (3) the collagen molecules inclining towards the adsorbing surface in all orientations comprised between 0° and 90°.

Desorption experiments performed on polymer surfaces (Figure 6) permit an insight into the structure of the adsorbed molecules. These experiments reveal that all collagen which adsorbs in addition to the irreversibly adsorbed layers (0.3 and 0.37 μg/cm², the maximum values on the polyethylene and the grafted polyethylene, respectively) can be entirely desorbed when the collagen containing solutions are replaced by the buffer solution. As the irreversibly adsorbed quantities are higher than the values of the "side-on" monolayer coverage (0.1 μg/cm²), the possibility of a "side-on" multilayer formation has to be ruled out, since then it could not explain why the detachment of adsorbed collagen molecules suddenly ends, leaving 3 or 4 monolayers at the surfaces. The second and the third hypotheses have one common point: the "end-on" position of the irreversibly adsorbed collagen molecules implies their attachment to the surface by one of their ends. In this case the amount of collagen molecules which can be accommodated in a close packed structure is equal to 25 μg/cm² which is about 70 times higher than the quantity obtained experimentally. This would mean that the adsorbed molecules are separated one from another and that much free space is left between them. If the adsorbed molecules were attached to the surface in a tilted "end-on" position (third hypothesis), the new collagen molecules arriving to the surface would find their place at the surfaces reduced and would rather tend to adsorb upon the sides of the already attached molecules. Such adsorption would favor a much easier desorption of collagen molecules which is experimentally observed. It seems therefore most probable that the irreversibly adsorbed collagen molecules are attached to the surfaces in a tilted "end-on" position. Such a tilted tree-like structure was imagined by other authors^{19,20} who measured only the irreversibly adsorbed amounts using ¹²⁵I collagen, and was later confirmed by Silberberg and Klein,²¹ using force-distance measurements.

The most realistic picture of the adsorption process that one may assume would be the model in which the loosely bound collagen molecules continue to adsorb onto the irreversibly adsorbed layer. The build-up of loosely bound collagen molecules is especially significant at higher protein concentrations in solution. The existence of a loosely bound fraction of adsorbed proteins is quoted in the literature,^{17,18} and was recently evidenced for mucin adsorption

at different surfaces, with the help of the same experimental technique as used in this study.^{11,16}

The slopes of the lower parts of the desorption-adsorption curves (Figure 6), equaling 0.2 and 0.05 for polyethylene and grafted polyethylene, indicate that the collagen interactions with the grafted surface are much stronger. Not only are these interactions stronger, but also the build-up of the loosely bound fraction is enhanced by the chemical modification of the polyethylene surface (Figure 5) through grafting.

In our previous studies of protein adsorption on the surface oxidized polyethylene,^{11,22,23} it was demonstrated that functional groups, when created at the surface of a hydrophobic polymer and when oriented upright to the polymer plane, enhance protein adsorption. The same phenomenon is observed for the polyethylene grafted with poly(maleic acid) by the procedure of the free radical polymerization as used in this study. In our earlier studies on wettability and calcium adsorption studies of the maleic acid grafted polyethylene films we have shown that the grafted carboxylic groups are mainly distributed at the surface of the polyethylene.^{14,15} This is confirmed here by the increased adsorption of collagen molecules. A similar increase of adsorbed protein on modified silicone surfaces was reported by Holly and Owens.²⁴ The hydrophilicity increase in this case was provided by the introduction of hydroxyl-groups along the siloxane polymer chains.

To analyze the kinetics of collagen adsorption at the studied interfaces we have used two models proposed by Bornzin and Miller²⁵ for either reversible or irreversible protein adsorption. As the experimental results of adsorption during the first 45 minutes corresponded only to the irreversible protein adsorption hypothesis the diffusion coefficient of collagen adsorption at the studied surface was calculated from the equation:

$$A = 2 c(D/\pi)^{1/2}(t)^{1/2}$$

where A is the protein surface concentration, c is the protein concentration in solution, D the diffusion coefficient, and t the adsorption time.

For a given protein concentration, the slope of the curve A vs. $t^{1/2}$ is used to determine the diffusion coefficient D .

Figure 7 shows that the collagen diffusion coefficient is concentration dependent and that for all studied surfaces the numerical values are almost the same. They vary between 0.3×10^{-8} and 12.0×10^{-8} $\text{cm}^2.\text{sec}^{-1}$ within the studied concentration range. These values are in good agreement with the value found by Penners et al.²⁰ for irreversibly adsorbed collagen on glass surfaces (0.6×10^{-8} $\text{cm}^2.\text{sec}^{-1}$).

Obrink²⁶ measured collagen diffusion coefficients by a light scattering method within the concentration range of 0.8–3.2 mg/mL (20°C, phosphate buffer pH = 7.5, ionic strength 0.15 M). His extrapolation of the diffusion coefficient curve to zero collagen concentration yielded the value of 7.8×10^{-8} $\text{cm}^2.\text{sec}^{-1}$ which was considered as the intrinsic collagen diffusion coefficient value. Our results obtained within the collagen concentration range of 0.005 – 0.05 mg/mL (Figure 7) correspond with this extrapolated part of Obrink's curve. It can also be noted from our results that decreasing collagen concentration brings about a rapid rise of the D values.

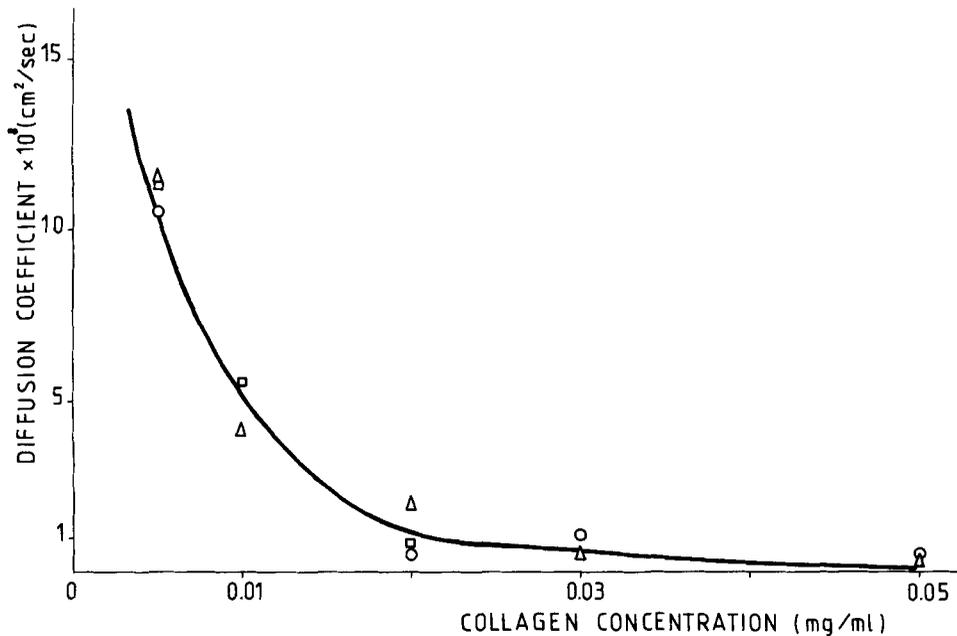


Figure 7. Concentration dependence of diffusion coefficient of collagen. Δ air; \square polyethylene; \circ poly(maleic acid) grafted polyethylene.

Two additional observations can be made in connection with the data represented in Figure 7. First, the adsorption of collagen from the buffer solution on the different studied surfaces followed the mechanism of diffusion-controlled adsorption in the absence of any electrostatic or steric barrier that might have hindered the process after the first protein molecules were adsorbed. Second, the concentration dependence of the diffusion coefficient can be attributed to the aggregation of collagen molecules in solution. A similar shape of the concentration dependence curve of the diffusion coefficient was found by us for adsorption of mucin on mica surfaces.¹⁶

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