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SiO_2 Entrapment of animal cells. Part 2:† Protein diffusion through collagen membranes coated with sol–gel SiO_2

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Membranes of collagen on a paper scaffold were manufactured to a thickness of 0.64 ± 0.03 mm and reacted with gaseous silicon alkoxide precursors until a 0.1 μm deposit of sol–gel SiO_2 was achieved. Diffusion of protein macromolecules myoglobin, albumin, γ-globulin and fibrinogen in an aqueous solution through these membranes was studied; kinetic runs for coated and uncoated SiO_2 were performed under experimental conditions suitable for calculating relevant diffusion parameters. The data show linear lowering of the diffusion coefficient with molecular size, the effect being enhanced by the deposition of the SiO_2 layer, which behaves as an effective barrier to the diffusion of high molecular weight protein macromolecules.

Introduction

Advanced proposals in designing bioartificial organs refer to cell encapsulation by materials which can maintain cell functionality, ensure mechanical resistance and produce a molecular cutoff excluding basic humoral immunological macromolecules.[2,3] The latter factor is crucial for hybrid bioartificial organs based on allogenic or xenogenic cell encapsulation, although it may imply important limitations to free mass transfer, with consequent inefficient cell functionality. For this purpose, the encapsulating material should have high porosity resulting from a pore size population not amenable to the classic Gauss distribution, which in any case characteristically has a pore fraction within the reach of immune-response macromolecules.[4] Materials with a regular crystalline habit, *e.g.* zeolites, may fulfil this requirement: however, porous crystalline materials have poor mechanical features and are usually prepared by methods unsuitable for living cell encapsulation.

Sol–gel SiO_2 deposited on cell surfaces from gaseous alkoxide precursors[5,6] appears to meet most properties required of an encapsulating material.[7] Chemical inertness, high mechanical strength and microarchitectural variety, producing tailored porosity, are some outstanding features inherent to sol–gel SiO_2.[8–11] The advantages of SiO_2 formation from gaseous precursors[12] consist of the possibility of controlling the thickness of the SiO_2 layer by reaction time and alkoxide partial pressure, the immediate elimination of toxic byproducts, and the deposition of a homogeneous SiO_2 layer in a single operation. This cell entrapment process has been tested during the design of a bioartificial liver, devised particularly to preserve hepatocyte function,[13–16] in a planar structure above or inside a collagen layer. Deposition of a 0.1–0.3 μm SiO_2 layer *via* reactive alkoxides from a flowing phase was preliminarily found to preserve the viability and functionality of adjacent hepatocytes in collagen.[17] A definite increase in elastic modulus and resistance to failure was also demonstrated for deposition of a 0.1 μm SiO_2 layer on silk fibers, mimicking a natural protein material.[1] In an effort to define the possible molecular cutoff inherent in these sol–gel SiO_2–collagen layered structures, we report here a diffusion study on protein macromolecules, with molecular weights in the range 17 800–341 000, across membranes of collagen coated with sol–gel SiO_2.

†Part 1: ref. 1.

Experimental

Preparation of membranes

Paper disks (100 μm thick and 28 mm diameter) were cut from sheets produced by Schleicher & Schuell (cellulose nitrate filter paper, pores 0.45 μm in diameter), placed on glass plates (10 mm thick and 30 mm diameter), treated with collagen extracted from rat tails[18] and used in a 1.1 mg cm^{-3} solution of 0.1% acetic acid. To prepare the collagen membranes on the paper disks, 8 parts of the collagen solution were treated with one part of Ham's F 12 10X medium (Sigma) and one part of 0.34 M NaOH: 300 μl of this mixture were spread on the paper and left at 37 °C for 2 h in a vapor-saturated incubator.[19] Optical microscopy observations of some membranes, cut perpendicularly to the external surface, indicated the homogeneous deposition of collagen in a layer 0.64 ± 0.03 mm thick. Single membranes were removed from the glass plates and placed between the terminal rings of glass compartments A and B (volume = 20 cm^3) (Fig. 1), care being taken to ensure that the collagen surface was oriented toward the interior of A. Compartments A and B were fixed by metallic pliers and the joint was sealed by silicone grease. Membrane integrity and positioning were checked by direct observation with a magnifier.

Sol–gel SiO_2 deposition was carried out on membranes

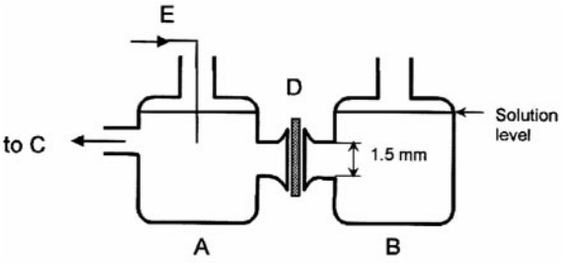

Fig. 1 Schematic diffusional apparatus: A = donor compartment, B = receptor compartment, C = reservoir, D = membrane, E = needle inlet.

already assembled between compartments A and B. According to the reported procedure,[1] a solution of $Si(OEt)_4$ (Petrarch System) and $HSiCH_3(OEt)_2$ (Petrarch System) in 80 : 20 molar ratio was bubbled at 80 °C with dry air, providing a flux of 400 cm^3 min^{-1}. The flux was directed to container A by metallic needle E and treatment was applied for 60 s: considering the exposed collagen surface, the partial pressure of the silicon alkoxides, and the total flux, this period of time was estimated to produce a 0.1 μm layer of nominal SiO_2 on the collagen surface.[1] Containers A and B were filled with a buffer solution [0.137 M NaCl–0.0027 M KCl–0.01 M Na_2HPO_4 (pH = 7.4, ionic strength = 0.1617 M)].

Before each protein diffusion experiment, sodium azide (NaN_3, Aldrich) was added to compartments A and B (Fig. 1) up to 0.02 wt%, to prevent biological contamination of the protein solution.

Some SiO_2 coated membranes were further treated with gaseous silicon alkoxides in the above-described conditions; before each treatment, the membrane, assembled as shown in Fig. 1, was kept for 10 min in a buffer solution at pH = 7.4 and left to dry for 12 h at room temperature.

For scanning electron microscopy (SEM) observations, the sol–gel SiO_2 layer was deposited on the collagen directly on the glass plates, adjusting the experimental conditions to obtain a 0.1 μm SiO_2 deposit.

Diffusion kinetics

Container A (Fig. 1) was connected to reservoir C, filled with the same solution having a volume 20 times larger than compartments A or B; a peristaltic pump provided continuous circulation between A and C across port C and needle inlet E. The apparatus was thermostated at 25 ± 1 °C by immersion in a water bath, care being taken to maintain the same level of solution between compartments A and B, operating on the flexible joint to port C. A weighed amount of each protein was introduced into compartment A and, after 10 min circulation with port C, a 0.2 cm^3 sample of solution was taken from compartment A to determine the initial concentration, C_A^0, of the diffusant protein.

Diffusion kinetics were followed by collecting 8–10 samples of solution from container B within a 2–3 day interval. The final samples were simultaneously taken from both containers A and B, to check the final concentration in compartment A in comparison with the initial one.

Diffusion experiments on each protein were carried out in duplicate: one for the membrane composed of collagen alone, and one for the collagen membrane coated with sol–gel SiO_2. The protein concentration of each sample was determined by HPLC (Jasco, PU-980, diode array mod. MD 910, $\lambda = 210$ nm), equipped with a HPLC Sec 1000/17 Biorad column, with an eluting solution of 19 mM KH_2PO_4–30 mM K_2HPO_4–150 mM NaCl, with a flux of 1 cm^3 min^{-1}. A calibration curve was previously determined, leading to an instrumental reliability of 2% in the protein concentration range 0.2–30 μM.

Kinetic experiments were carried out for myoglobin (MW 17 800), albumin (MW 67 000), γ-globulin (MW 150 000) and fibrinogen (MW 341 000) (all >95% purity from Sigma).

The stability of these macromolecules in the buffered solution, treated with NaN_3, was checked for 48 h, and was found to be comparable with the 2% instrumental uncertainty.

The optimization of diffusion parameter calculations was performed by using a Fortran 77 program, based on least square root method. Fourier expansions, relevant to considered diffusion models, were restricted to 1/10 000 difference between two consecutive terms. The double precision approach was used for calculations.

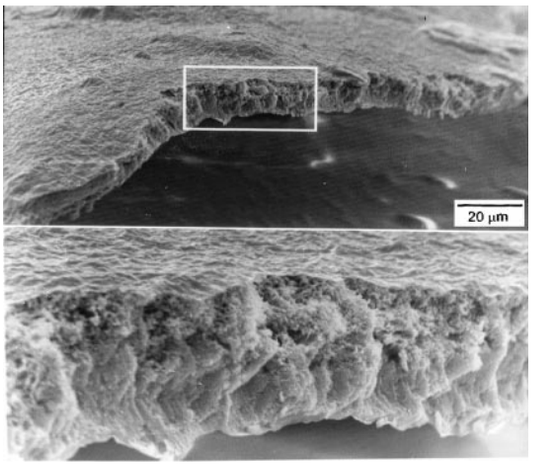

Fig. 2 SEM images of a collagen layer coated by sol–gel SiO_2. Collagen shrinkage occurred with the sample preparation under reduced pressure so that the original 0.64 mm thickness is reduced to *ca.* 20 μm; dark regions correspond to the glass plate surface.

Results

Membrane characterization

The thickness of the collagen gel layer (0.64 ± 0.03 mm) was calculated from the volume of the solution deposited on the paper disk used as scaffolding material. This value, confirmed by optical microscopy, was chosen as reliable for complete encapsulation of layered hepatocytes, and includes the thickness of the paper imbued with the original collagen solution. As shown in Fig. 2, the sol–gel SiO_2 from gaseous precursors appears to be homogeneously deposited and denser than the collagen substrate. A thickness of 0.1 μm SiO_2 was achieved by adjusting the known dependence of SiO_2 deposition on exposure time for a given alkoxide flux,[1] and was confirmed by transmission electron microscopy.[20]

Subsequent depositions of SiO_2 were carried out under the same experimental conditions as previously, the exposed surface being exhaustively hydrolyzed before reaction; the thickness of 0.1 μm, referring to a single treatment, probably increases linearly with the number of treatments.

Diffusion kinetics and data processing

Fig. 3 shows representative plots of the amount of diffused protein *vs.* time. The protein quantity was measured by HPLC analysis of samples taken from container B, since the protein in

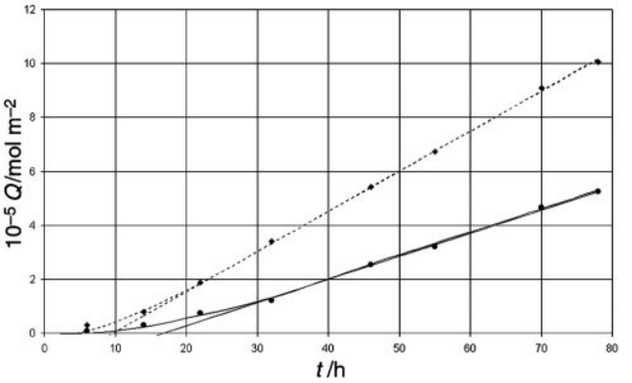

Fig. 3 Protein diffusion through different membranes: (···) γ-globulin across the collagen membrane; (—) fibrinogen across the SiO_2 coated collagen membrane. The curves represent the best fit of the general Daynes–Barrel equation to the experimental data.

Table 1 Diffusion coefficients calculated by different methods

Protein	$10^{12}\ D^a$/m^2 s^{-1}	$10^{12}\ D^b$/m^2 s^{-1}	$10^{12}\ D^c$/m^2 s^{-1}
Myoglobin (MW = 17 800)			
SiO_2 + collagen	2.5 ± 0.3	2.5 ± 0.3	2.6 ± 0.3
collagen	2.6 ± 0.3	2.6 ± 0.3	2.7 ± 0.3
Albumin (MW = 67 000)			
SiO_2 + collagen	2.0 ± 0.2	2.1 ± 0.2	2.1 ± 0.2
collagen	2.3 ± 0.2	2.3 ± 0.2	2.4 ± 0.2
γ-Globulin (MW = 150 000)			
SiO_2 + collagen	1.6 ± 0.2	1.6 ± 0.2	1.7 ± 0.2
collagen	2.1 ± 0.2	2.1 ± 0.2	2.2 ± 0.2
Fibrinogen (MW = 341 000)			
SiO_2 + collagen	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
collagen	1.9 ± 0.1	2.0 ± 0.1	2.2 ± 0.1

[a]From lag time. [b]From best fitting with the Daynes–Barrel equation. [c]From the Paul–Dibenedetto treatment.

diffusant containers A + C remains virtually constant during the kinetic run, owing to the volumetric ratio B/(A + C) = 0.048. This experimental condition fulfils the requirement $C_A^0 = C_A^t$ (C_A^0 = concentration of protein in container A at time $t = 0$) to solve Fick's second law in $t_L = h^2/6D$, according to Daynes and Barrel[21] (t_L = lag time, h = thickness of the planar isotropic membrane, D = diffusion coefficient), although the additional requirement $C_B^t \approx C_B^0 = 0$ was not observed. Owing to the latter limitation, plots of the type shown in Fig. 3 were used to calculate D according to the following data processing improvements:

(i) Lag time was calculated as an intercept on the time axis of the straight line drawn for experimental points taken above $t = 2.7\ t_L$ (steady-state condition),[22] providing an evaluation of the standard deviation on t_L by ordinary best fitting treatment. The diffusion coefficient D was roughly evaluated from $D = h^2/6t_L$, the standard deviation error on D resulting from uncertainties in t_L and h (0.64 ± 0.03 mm). Values of D obtained are listed in Table 1.

(ii) Subsequently, direct calculation of D by linear interpolation of the steady-state experimental points of each kinetic run was made with the Daynes–Barrel equation $Q = [(DKC_A^0 t/h) - (KC_A^0 h/6)]$[21,22] ($K$ = partition coefficient between protein solution and collagen membrane, Q = cumulative quantity of diffused protein per unit area, considering a constant surface area = 1.77×10^{-4} m^2). As coefficient K cannot be independently measured, it was considered as a fixed parameter for both coated and uncoated SiO_2 membranes. Best fitting calculations gave $K = 22.4 \pm 0.5$ for all experimental data, irrespective of protein structure or bulkiness. Representative examples of the best fits of the above equation to the experimental points are shown in Fig. 3. The same value of partition coefficient K was obtained from optimization of D values by best-fitting all the experimental points of each kinetic run with the general equation.[21,22] The resulting D values are listed in Table 1.

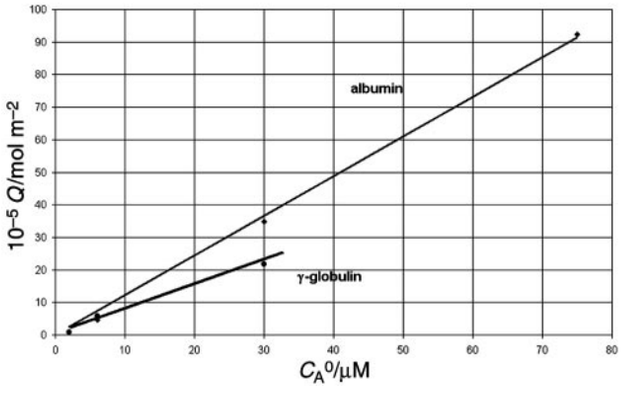

Fig. 4 Cumulative quantity of diffused protein, Q, after 48 h as a function of the concentration in the donor compartment, C_A^0, for collagen membranes.

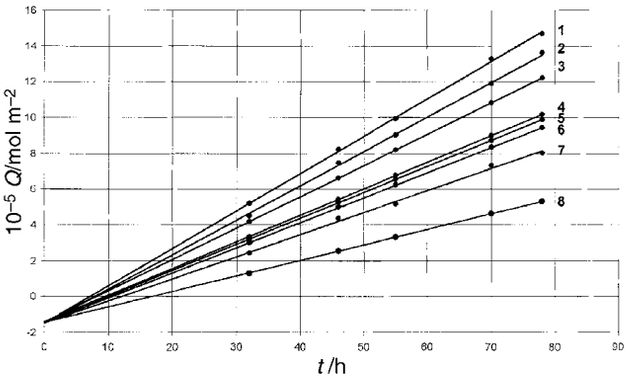

Fig. 5 Plots of Q *vs.* time: (1) myoglobin, (3) albumin, (4) γ-globulin and (6) fibrinogen across collagen membranes; (2) myoglobin, (5) albumin, (7) γ-globulin and (8) fibrinogen across SiO_2 coated membranes.

(iii) The Paul–Dibenedetto[23] approach was used for calculating D: this model takes into account that $C_B^t \neq C_B^0 = 0$, although it agrees with the Daynes–Barrel solution, as the membrane volume can be considered negligible in comparison with the volume of B. Best fitting by regression analysis of experimental data with the Paul–Dibenedetto equation of Q[23] gave the D values shown in Table 1. The coincidence of the results obtained by this treatment of data with that obtained according to method (ii) is of note.

Best fits on both Paul–Dibenedetto and Daynes–Barrel models were achieved with a Fortran non-linear regression program.

The D values shown in Table 1 were obtained using a protein concentration $C_A^0 = 6.0$ μM; for γ-globulin (MW = 150 000) and albumin (MW = 67 000), the independence of D with C_A^0 was demonstrated by the linear relationship between Q and C_A^0 values at $t = 48$ h (Fig. 4). This fact indirectly confirms the validity of the Daynes–Barrel solution (ii) for the calculation of diffusion coefficients.

Kinetic experiments carried out for $C_A^0 = 6.0$ μM fibrinogen solutions with membranes with SiO_2 deposits obtained after two or three treatments did not give reliable D values, since the measured C_B^t concentrations for $t = 44$ and 70 h were still within the HPLC instrumental detection limit.

Discussion

The membranes studied here resemble the Okahata capsule membranes composed of an ultrathin nylon matrix corked with porous lipid layers.[24] In our case, collagen provides a soft macroporous protein network, and sol–gel SiO_2 constitutes a mechanically strong thin microporous deposit. As both collagen and SiO_2 layers are hydrophilic, a single mass transfer mechanism may be expected for membranes prepared with these materials. Calculation of diffusion coefficients (Table 1) is in effect based on the assumption that the membrane behaves as an isotropic medium, a statement which may be contested since collagen is coated by a not strictly isotropic SiO_2 layer. Accordingly, standard treatments of diffusional curves to determine lag times and D values require some comment and specifications.

We found that plots of Q *vs.* time (Fig. 5), under steady-state conditions, converge at $t = 0$ to $Q = -1.44 \times 10^{-5}$ mol m^{-2}, irrespective of the SiO_2 coating. This observation suggests that the SiO_2 layer on the collagen membrane does not affect either the value of h [in line with the negligible contribution of the SiO_2 deposit (0.1 μm) to the collagen thickness (0.64 ± 0.03 mm)] or partition coefficient K. With these assumptions, and the observation that the D values of Table 1 are independent of C_A^0 (Fig. 4), total diffusional

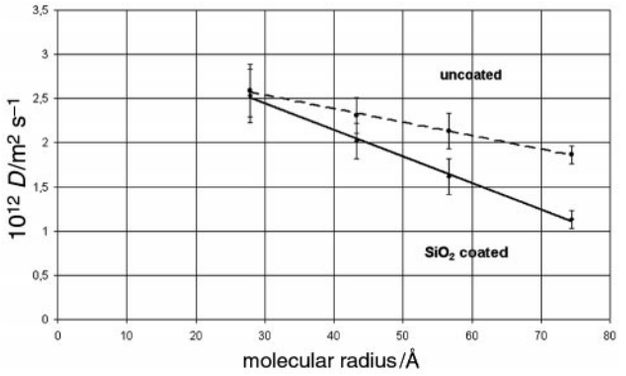

Fig. 6 Protein molecular dimension dependences of diffusion coefficients of uncoated and SiO_2 coated collagen membranes.

resistance $R_T = h_T/D_T K$ may be computed according to the model of Flynn and Yalhowsky:[21,25]

$$\frac{h_T}{D_T K} = \frac{h_C}{D_C K} + \frac{h_{Si}}{D_{Si} K} \quad (1)$$

where T, C and Si indicate total, collagen and SiO_2, respectively. Eqn. (1) is based on the assumption that the individual layers contribute as independent and additive resistances to total resistance; moreover, the resistances of the aqueous region at the receptor and donor sides of the membrane are not explicitly expressed. Substitution in eqn. (1) of $h_T = h_C = 0.64 \pm 0.03$ mm, $h_{Si} = 0.1$ μm, D_T and D_C values (Table 1) allows the calculation of D_{Si} for the diffusion of the four proteins examined here. The resulting D_{Si} values are $2.6 \pm 2.8 \times 10^{-15}$ m^2 s^{-1} (albumin), $1.1 \pm 0.8 \times 10^{-15}$ m^2 s^{-1} (γ-globulin) and $0.34 \pm 0.08 \times 10^{-15}$ m^2 s^{-1} (fibrinogen). The D_{Si} value calculated for myoglobin is unreliable.

Although eqn. (1) is limited by the demanding collection of experimental data and may be affected by the approximation of the real diffusional situation to ideal models, it is remarkable that our roughly calculated D_{Si} values decrease by more than one order of magnitude from albumin to fibrinogen. This fact highlights the significant reduction in diffusion characterized by the sol–gel SiO_2 deposit for proteins having bulkiness greater than that of albumin, *i.e.* most immunoglobulin macromolecule components.

The D_{Si} values fit the diffusion coefficients reported for protein macromolecules; D (myoglobin) $= 1.33 \times 10^{-10}$ m^2 s^{-1} and D (fibrinogen) $= 1.97 \times 10^{-11}$ m^2 s^{-1} are quoted at 25 °C in pure water:[26] the one order of magnitude difference is similar to the difference between our D_{Si} values. The exceptionally low D_{Si} values are not surprising. Diffusion coefficients with magnitudes of the order of 10^{-12} m^2 s^{-1} have been reported for steroid (progesterone) diffusion in sol–gel SiO_2 of average pore diameter 30 Å,[27,28] and reduction by some orders of magnitude has been found as the molecular bulkiness increases. It is evident that D_T (Table 1) and D_{Si} values are strongly affected by the molecular dimensions of the diffusing species. In general, this dependence is expected to be inversely proportional to the protein dimension, r, which can be calculated as the cube root of the molecular volume (obtained in Å^3 by multiplication of the molecular weight by 1.21), this parameter resulting from the average of literature data.[29,30]

Comparison between diffusion across SiO_2 coated and uncoated membranes as a function of protein dimension r is shown in Fig. 6. The 0.1 μm sol–gel SiO_2 layer does not appreciably change myoglobin diffusion through the composite membrane, but does emphasize the decrease in linear diffusion with increased protein size. This feature explains the absence of fibrinogen diffusion through collagen membranes coated with two or three layers of sol–gel SiO_2: nominal h_{Si} values of 0.2 or 0.3 μm with reported D_{Si} and D_T values lead to calculated t_L values too large for reliable collection of data with the experimental approach used here.

In conclusion, this work highlights the significant effect of a very thin SiO_2 deposit on a collagen layer to reduce the diffusion of protein macromolecules. The dependence of the diffusion coefficient on SiO_2 thickness, although only roughly ascertained, does indicate a substantial reduction in the rate of diffusion for proteins having MW > 150 000. This fact may represent a useful basis in designing a bioartificial liver set-up with collagen-entrapped xenogenic or allogenic hepatocytes, since the immunological response is strongly retarded.

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