Crosslinked Poly(ethylene glycol) Networks as Reservoirs for Protein Delivery

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SYNOPSIS

Poly(ethylene glycol)s are cross-linked by tris(6-isocyanatohexyl)isocyanurate via urethane/allophanate bond formation to obtain polymeric networks capable of swelling in phosphate-buffered saline or ethanol, resulting in gels. The protein loading of the gels swollen in ethanol is enhanced by formation of hydrophobic ion-paired complexes of proteins with sodium dodecyl sulfate. The protein release profile from loaded gels through biomimetic membranes impregnated with phosphatidylcholine is governed by the gel crosslinking density. Spectroscopic data and enzymatic activity of the proteins released from the gels into aqueous solutions indicate that proteins are recovered unchanged upon their redissolution in water. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

Biocompatibility of poly(ethylene glycol) (PEG) makes it the polymer of choice for numerous biomedical applications.[1] Graham and co-workers[2] pioneered the field of PEG networks crosslinked by diisocyanates as reservoirs for drug delivery. They explored loading of low molecular weight compounds into and release from PEG hydrogels. In this study, PEG networks are challenged as reservoirs for delivery of macromolecules, such as proteins, via a transdermal route. The rate-limiting step of transdermal drug delivery is the diffusion through the structured lipids located in the intercellular channels of the stratum corneum.[3] It may therefore be necessary to alter the structure of these lipids in order to use a transdermal path for delivery of large molecules. The use of ultrasound or benign penetration enhancers may appear to be a means to improve skin permeability. Recently, the hypothesis was forwarded that penetration of proteins through the skin can be achieved if the protein-containing medium would solubilize lipid barriers.[4] In other words, a protein-dissolving solvent should also behave as a penetration enhancer. Significant protein transport through a biomimetic membrane impregnated with phospholipid was observed from ethanol but not from water.[4] Recently, the role of ethanol as a penetration enhancer in percutaneous absorption and transport of nitroglycerin[5] and indomethacin[6] in biphasic transdermal delivery *in vitro* was emphasized. Thus, ethanol appears to be an important component of a reservoir in the transdermal system. On the other hand, the unique incompatibility of PEG with proteins in aqueous solutions[7,8] which stems from the dominant steric repulsion force between hydrated PEG coils and protein molecules[8] may be overcome by simply replacing water with nonaqueous solvent, thereby allowing higher protein loadings into the PEG gels. Therefore, this study primarily focused on the performance of PEG gels in neat ethanol.

EXPERIMENTAL

Materials

Poly(ethylene glycol)s (PEGs) of M_r below 20,000 were obtained from Aldrich; PEG of M_r 20,000 was supplied by Polysciences, Inc. PEGs were agitated under vacuum at 80°C overnight and stored in sealed flasks under nitrogen. Equivalents of hydroxyl groups in dried PEGs were determined as described elsewhere.[9] 1,6-Diisocyanatohexane (DIH, Aldrich, 98%) was distilled under vacuum and stored under

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nitrogen prior to use. Tetrahydrofuran (Aldrich, 99.9%, anhydrous) was stored under nitrogen before use. Dibutyltin dilaurate (95%), triethanolamine (98%), tri-*n*-butylphosphine (97%), and *n*-butylamine (99%) were all obtained from Aldrich and stored over molecular sieves. Bovine pancreatic Zn^{2+}–insulin (27 units/mg), hen egg-white lysozyme (52,000 enzyme units per mg of protein), myoglobin from horse heart (essentially salt free), egg yolk L-α-phosphatidylcholine (type X-E), phosphate-buffered saline (PBS) tablets, and sodium dodecyl sulfate (99%) were all obtained from Sigma. Proteins were lyophilized prior to use from their 5 mg/mL solutions in deionized water adjusted to pH 3.0.[4] All other chemicals were obtained from commercial sources and were of highest purity available.

Syntheses

Tris(6-isocyanatohexyl)isocyanurate (TIHI, crosslinking agent) was synthesized by trimerization of DIH as follows: A mixture of 168 g (1 mol) DIH and 1.0 g (6.7 mmol) triethanolamine loaded into a three-necked flask was purged by N_2 bubbling overnight. The stirred mixture was heated to 250°C over 5 min and then allowed to slowly cool down to room temperature. The resulting mixture was purified by repeated thin-layer distillation to afford TIHI of 23 wt % NCO content, as measured by chemical titration with *n*-butylamine,[10] and a DIH content below 0.5 wt %. IR (CH_2Cl_2): 2277 (*s*, N=C=O asymmetric stretching vibrations), 1689 (*m*, C=O in isocyanurate ring), 1466 (*m*, OC—NR=CO in isocyanurate ring).

PEG networks were synthesized as follows: To a flask containing a stirred solution of 1.0 g PEG and 0.2 wt % dibutyltin dilaurate in dry THF at 70°C a weighed amount of TIHI was added under nitrogen. The amount of THF was set such that a 10 wt % PEG resulted in the reaction mixture. The flask was agitated, sealed, and kept unperturbed at 70°C for 3 days. In some cases, formation of transparent gels without free THF was observed in the flasks, depending on the amount of TIHI in the reaction mixture. Gels were dried under vacuum for 2–3 days at 70°C until constant weights were maintained. The resulting white, dry PEG networks were cut to cubic-shaped pieces of 5–15 mg and stored in sealed vials.

Procedures

In swelling experiments, dry PEG networks of known dry weights (W_d) were placed in excess physiological phosphate-buffered saline (PBS) (pH 7.4) or absolute ethanol and allowed to swell for 2–3 days at room temperature until constant weights (W_s) were reached. The swelling degree was calculated by $(W_s/W_d - 1) \times 100$.

Two-compartment Teflon diffusion cells were used for membrane-transport experiments. The compartments had a volume of 15 mL each and were separated by a composite membrane (see below) with a working area of 3.14 cm^2. The *feed* and *receiver* compartments were initially filled with the species to be transported and aqueous solution, respectively. PEG networks were shredded into small pieces of 3–7 mg and swollen in protein-containing PBS or ethanol at 37°C. These pieces containing a known amount of protein were loaded into the feed compartment. At certain time intervals, samples were withdrawn from the receiver compartment and, after the removal of turbidity by centrifugation (28,000*g*, 0.5 h), were analyzed for the protein content with the bicinchoninic acid (BCA) assay.[11] The ethanol content was assayed by gas chromatography.

The solubility of a protein in ethanol was measured by placing it in a screw-cup vial, followed by addition of the solvent. The resultant suspension was shaken at 37°C for a day and then centrifuged at 28,000*g* for 0.5 h. The undissolved residue was removed, and the supernatant was evaporated to dryness under vacuum. The resultant solid was redissolved in PBS, and the protein concentration was determined by BCA assay. Separate tests demonstrated no interference of SDS in the protein determination.

Cumulative release of protein or ethanol was calculated by $\sum C_t/C_\infty \times 100$, where C_t is the concentration in the receiver phase measured at time t and C_∞ is the concentration attainable in the receiver compartment at equilibrium. Precipitates and liquid samples were analyzed for phospholipid content by elemental analysis for phosphorus. Phospholipid removal from the membrane was calculated by $\sum [P]_t/[P]_m \times 100$, where $[P]_t$ is the phosphorus content in the receiver phase measured at time t and $[P]_m$ is the phosphorus content in the amount of phosphatidylcholine corresponding to that used for the membrane impregnation (see below).

The activity of lysozyme transported into the receiver chamber was measured spectrophotometrically on the basis of the enzymatic lysis of the dried cells of *Micrococcus lysodeikticus*,[12] after dialysis of the corresponding sample for 48 h at 4°C against 66 m*M* potassium phosphate (pH 6.24) buffer. After withdrawal of the sample from the receiver compartment, the latter was immediately filled with

fresh aqueous solution. The receiver compartment was vigorously stirred with a magnetic stirring bar. All transport experiments were conducted at 37°C.

A composite membrane of a total thickness of 0.4 mm was made of 589 black ribbon cellulose filter paper (Schleicher & Schuell) with a pore diameter of 20–30 mm, impregnated with phosphatidylcholine (phospholipid/cellulose ratio 2.2/1.0), and identical nonimpregnated membrane facing the receiver compartment. The process of the membrane impregnation was described in detail elsewhere.[4]

A Perkin-Elmer 1600 FTIR spectrometer was used to record all infrared spectra with a standard resolution of 2 cm^{-1} in a water-free atmosphere, applying NaCl crystals for liquid samples and thin films, and KBr tablets for solids. An Aviv 62DS spectrophotometer was used to measure all circular dichroism (CD) spectra with a bandwidth of 1 nm and an averaging time of 3 s at 25°C (Ref. 13) in strain-free fused silica cells of 1 mm path length.

RESULTS AND DISCUSSION

PEG Networks

Conventionally, swelling equilibria in gel/solvent systems are considered in terms of polymer–solvent interactions and gel network parameters, such as molecular weight between crosslinks and crosslink density, which define the elastic contribution from deforming the network chains from their reference state.[14] In the case of PEG gels, Graham et al.[9] stressed the effect of hydrogen bonding between solvent and PEG, and the PEG crystallinity, on gel swelling. Gander et al.,[15] however, demonstrated that loading of low molecular weight drugs and crosslinking significantly decrease the initial crystallinity of the PEG chains, so as other parameters, such as copolymer composition and interlinking degree, influence the gel swelling. Following earlier works,[2] they employed pairs of polyols (branching agents) and diisocyanates to crosslink PEGs and thus expressed the interlinking degree as mol of branching agent per mol of initial polymer. The stoichiometry of crosslinking, however, in these multicomponent systems is extremely complicated, so that the interlinking degree can hardly be related to the gel structure. In this study, trifunctional isocyanate (isocyanurate) is used as a model crosslinker, thereby eliminating the need for a branching agent. Network structure is characterized by the initial ratio of equivalents of isocyano and hydroxyl groups ([NCO]/[OH]) set for the gel preparation and by molecular weight between crosslinks (MW).

Homogeneous gelation of the entire reaction mixture (see Experimental) was observed in the region $0.65 < [NCO]/[OH] < 3.4$ for all PEGs studied. Outside this region, the mixtures remained liquid, sometimes with substantial precipitate formed. Figure 1 shows infrared spectra of the original TIHI and reaction mixtures containing different [NCO]/[OH] ratios. PEG of MW 3400 was used throughout. A mixture of [NCO]/[OH] = 11 [Fig. 1(B)] resulted in the liquid, whereas [NCO]/[OH] of 1.0 and 0.65 [Fig. 1(C) and (D), respectively] yielded homogeneous gels.

It can be seen that the peak at 2277 cm^{-1} (unreacted NCO vibrations) which dominates spectra (A) and (B) disappeared as [NCO]/[OH] approached 1.0 or lower, giving rise to a peak at 1720 cm^{-1} (urethane bond vibrations) [spectra (C) and (D)]. This manifests formation of the urethane

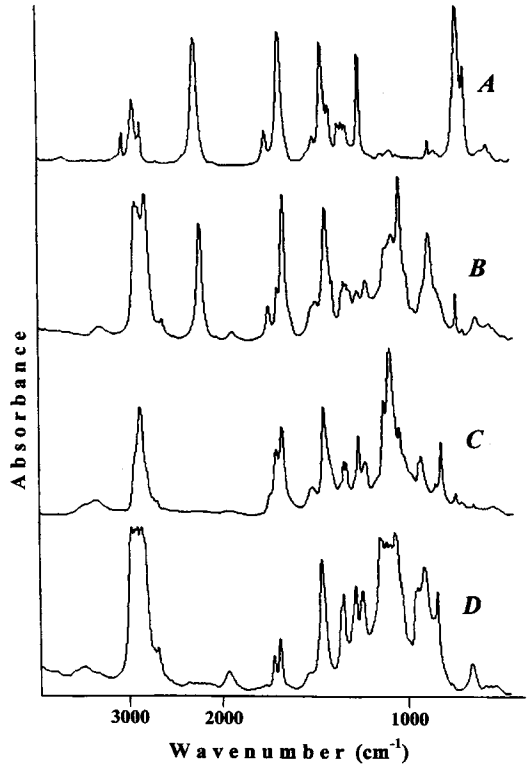
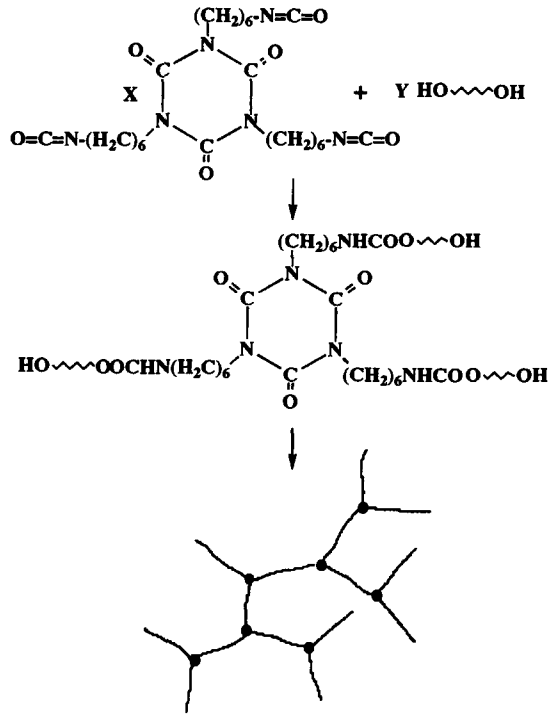
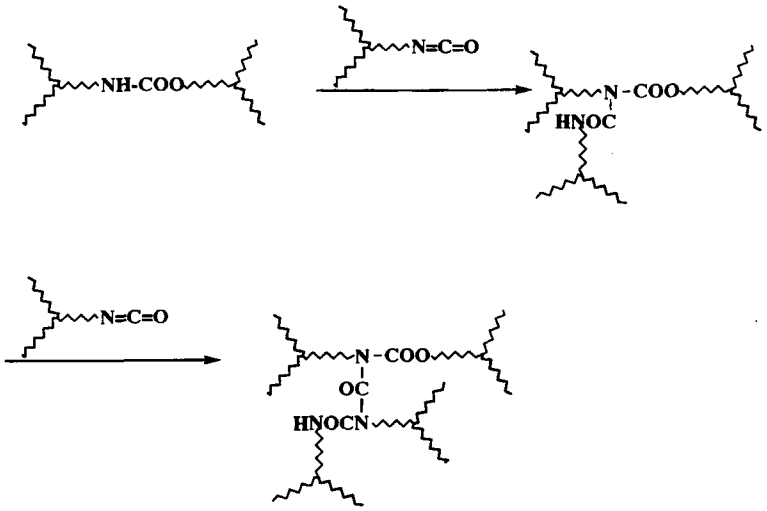


Figure 1 FTIR spectra of (A) tris(6-isocyanatohexyl)-isocyanurate (TIHI) in dichloromethane, (B) a mixture of PEG 3400 and TIHI that resulted in a liquid after reaction, [NCO]/[OH] = 11, (C) a mixture of PEG 3400 and TIHI that resulted in a gel after reaction, [NCO]/[OH] = 1.0, and (D) a mixture of PEG 3400 and TIHI that resulted in a gel after reaction, [NCO]/[OH] = 0.65. Spectra (A) and (D) were run in NaCl cells; gels in (C) and (D) were cast as thin films from THF onto NaCl crystals. For other experimental conditions, see Experimental.

crosslinks between PEG chains leading to a starlike polymeric network:

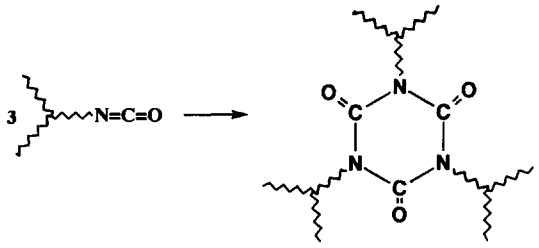


Cyclization via allophanate and urea bonding causes additional branching of the network, especially in the region [NCO]/[OH] > 1 (Ref. 16):



If traces of water or tri-*n*-butylphosphine (catalysts for isocyanate polymerization[17]) were introduced into the liquids resulting from reaction mixtures where [NCO]/[OH] > 3.4, the latter quickly hardened at elevated temperature, resulting in brittle transparent gels. Figure 2 shows infrared spectrum of the [NCO]/[OH] = 11 mixture [compare with the spectrum in Fig. 1(B)] gelled by introduction of 0.2 wt % tri-*n*-butylphosphine at 70°C.

It can be seen that unreacted NCO groups disappeared, while the amide II bond corresponding to fully substituted ureas increased (shoulder at about 1660 cm^{-1}). This represents yet another process of the network crosslinking via isocyanurate trimerization:



Since gels prepared with excessive amounts of isocyanurate (last scheme prevails) possessed unfavorable properties such as a low degree of swelling and severe brittleness, they were not included in this study.

Figure 3 illustrates the swelling of the PEG networks in PBS and ethanol as a function of the PEG molecular weight and composition of the initial mixture. It appeared that the swelling degree increases with the increasing molecular weight of the initial PEG (which is proportional to the molecular weight between crosslinks after gelation). It is inversely proportional to the [NCO]/[OH], which

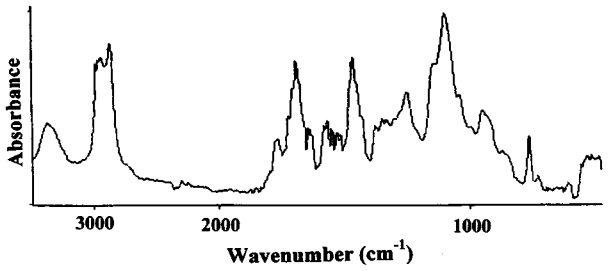


Figure 2 FTIR spectrum of the mixture of PEG 3400 and TIHI, [NCO]/[OH] = 11, gelled by introduction of 0.2 wt % tri-*n*-butylphosphine [compare with the spectrum in Fig. 1(B)]. Gel was dispersed in KBr.

reflects the crosslink density in the gel formed. The uptake of ethanol was higher than PBS uptake for the low MW PEGs (1000 or lower), whereas the opposite trend was observed for higher MW PEGs [compare Fig. 3(A) and (B)]. Similarly, Graham et al.[9] observed a higher uptake of water than of ethanol at room temperature for PEG with an M_n of 4200. Gander et al.[15] showed a reciprocal trend with water and benzyl alcohol for PEGs 600–6000 at 37°C. In our experiments, swelling of the gels in PBS at 37°C was 10–15% lower than at 25°C for PEGs 8000 and 20,000; no significant influence of temperature was detected for lower MW PEGs. A somewhat sharper temperature dependence of the gel swelling in pure water[9] may be attributed to the presence of salts in PBS in our case affecting the PEG–water association.

A detailed study of the gel swelling as a function of the PEG molecular weight at [NCO]/[OH] = 1.0 (Fig. 4) shows linear dependencies with deviation (lower swelling) for PEG 20,000. This deviation may be explained by the presence of lower MW fraction molecules in this PEG (data from the supplier) which resulted in higher crosslinking. It is interesting to note that Gander et al.[15] observed deviation from linearity (higher swelling) for the short-chain PEG molecules. They attributed this to the longer distance between the interlinking sites of the network composed of initially shorter-chain molecules than the distance of one single chain length (initially long-chain PEG). Substantial swelling of cross-linked PEG networks (Fig. 3) enabled consequent studies of protein loading and release to be carried out.

Protein Loading and Release

Loading of the gels was conducted at 37°C in batch conditions by shaking PEG gel of known dry weight with a protein dissolved in the *feed* solution (either water, pH 3.0, or ethanol) followed by gel removal and assaying of the *supernatant* for the protein content. The effective volume of the swollen gel (V_g) was measured by placing gel into a graduated cylinder. Effective gel loading was determined by the difference in protein concentrations in the feed and supernatant solutions. The kinetics of the loading was monitored for 3–5 days and only equilibrium data are further discussed. No precipitation of proteins took place. The partition coefficient (K_p) of a protein was calculated by $K_p = (V_f C_f - V_s C_s)/V_g C_s$, where V_f, C_f, and V_s, C_s are the volume and con-

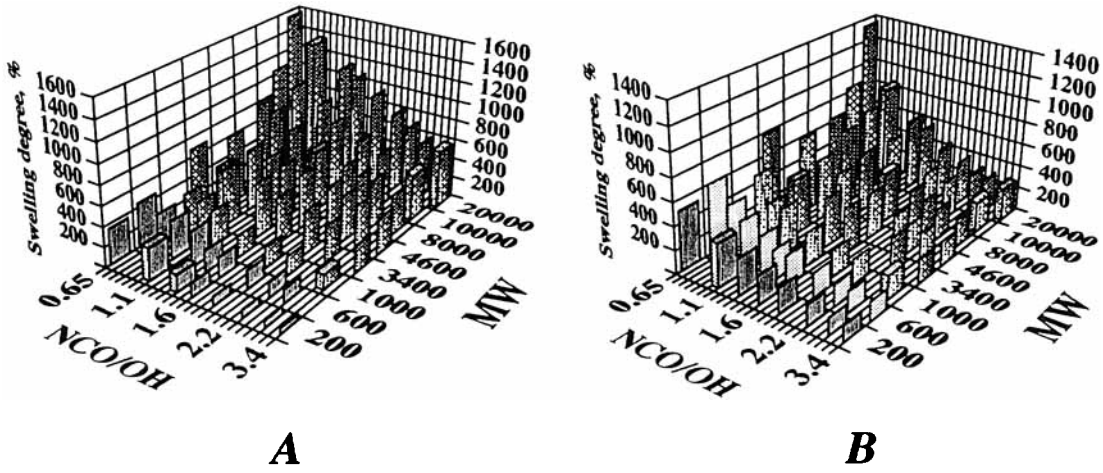


Figure 3 Dependencies of equilibrium swelling degree of gels in (A) PBS and (B) ethanol on the initial molecular weight of PEG (MW) and the ratio between equivalents of isocyano and hydroxy groups initially set up in the reaction mixture ([NCO]/[OH]).

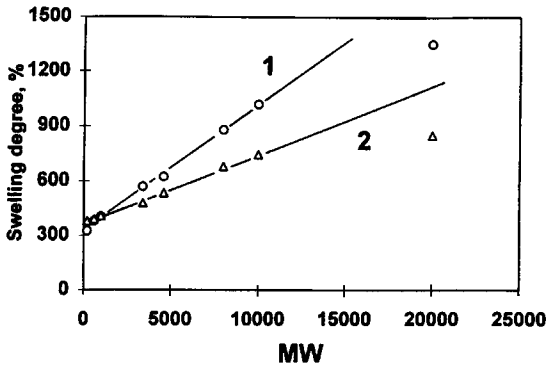


Figure 4 Equilibrium swelling degree of PEG gels in (1) PBS and (2) ethanol vs. MW at [NCO]/[OH] = 1.0.

centration of the feed and supernatant solutions, respectively.

Loading of proteins into PEG gels from aqueous solutions was observed to be low, irrespectively of the MW of the PEG. For instance, K_p did not exceed 0.05, 0.06, and 0.1 for myoglobin, lysozyme, and insulin, respectively, from 2 mg/mL of either protein in PBS. Apparently, the proteins were effectively excluded from the bulk of the gel, similar to the very well-documented protein exclusion from concentrated PEG solutions.[1,8] However, swelling of the PEG networks in 0.025 wt % protein solution in ethanol yielded a K_p of 1.0 and higher for each of the three proteins studied. A strong effect of network parameters, such as crosslinking density (as reflected by [NCO]/[OH]) and the chain length between crosslinks (MW) was observed: K_p values of 0.9, 0.8, and 0.5 were determined for the gels (PEG 20,000) of [NCO]/[OH] equal to 0.76, 1.0, and 3.4, respectively, from a 0.025 wt % ethanolic solution of lysozyme. The gel of PEG MW 1000 and [NCO]/[OH] = 1.0 afforded only K_p = 0.2 for lysozyme in the same conditions.

To achieve higher protein loadings, it is necessary to use either highly concentrated feed solutions or to apply repeated soaking of the gels in protein solutions and drying. Note, however, that the limited protein solubility in alcohols[4] almost prohibits the first of these two routes, unless it is enhanced by specific additives (see below). However, in the transport experiment, where the gel loaded with 5 mg insulin per mL gel by repeated soaking-and-drying followed by the gel reswelling in ethanol (swelling degree 1400%) constituted the feed phase of the membrane cell (see Experimental section), insulin release into the receiver compartment did not exceed 5% after 2 days, although phospholipid removal from the membrane was higher than 70%. This result is indicative of the strong PEG–protein complexation during the drying process which impedes protein release even after the gel reswelling in ethanol. Recently, it has been shown that freeze-drying of protein and PEG solutions leads to the formation of hydrophobic complexes, which, after being redissolved in ethanol, still behave as strongly associated species.[18] Hence, from the release standpoint, drying of the protein-loaded PEG gels should be avoided.

High protein loadings into the PEG gels without repeated soaking-and-drying could be achieved if gels were made to swell in concentrated ethanolic protein solutions. Therefore, it was necessary to enhance protein solubility in this solvent. To this end, a recently developed approach to protein solubilization in organic solvents via hydrophobic ion pairing with detergents[13,19] was applied. Since all the proteins studied are cationic at pH 3.0, cationic detergent, SDS, was employed to form hydrophobic complexes with the proteins. SDS concentrations used were always below critical micelle concentration.[4] To a solution of insulin, myoglobin, or lysozyme at 1 mg/mL in distilled water (pH 3.0), 1.7, 0.87, or 0.87 m*M* SDS, respectively, was added.[4] The ensuing precipitates were filtered, dried under air, and suspended in ethanol. Solubilities of at least 5 mg/mL were attained for all the proteins studied (for the solubility measurement methods, see Experimental). Consequently, ethanol-swollen PEG gels containing 0.2–0.3 wt % of each protein were obtained by placing dry networks into the excess of the corresponding 5 mg/mL protein–SDS solution in ethanol and shaking at 37°C. Insulin-loaded gels were subjected to membrane transport experiments where they served as a feed phase; the results are depicted in Figure 5.

The control experiment without a gel in the feed phase [Fig. 5(A)] showed a much faster release of the insulin than from the unstirred protein-containing gels [Fig. 5(B)–(D)]. Both protein and ethanol release reached the steady state after a certain lag time corresponding to the dissolution of the phospholipid by the ethanol leaching from the feed phase. When the feed phase was composed of a PBS-swollen, but otherwise identical, gel, loaded with insulin, the barrier properties of the phospholipid layer were not compromised, and thus no substantial protein release was observed [Fig. 5(C)]. Similar results have been reported with the biphasic transdermal systems where ethanol was employed as a penetration enhancer.[5,6] The rate of protein release was affected by both the crosslink density in the gel [compare Fig. 5(C) and (D)] and the molecular weight of PEG between crosslinks [compare Fig.

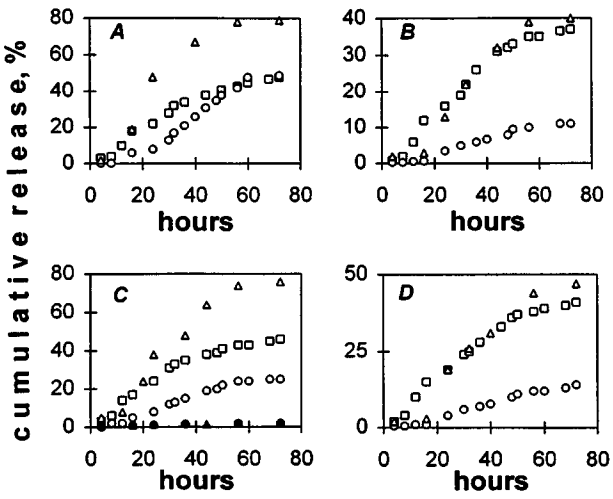


Figure 5 Kinetics of insulin and ethanol release from PEG gels ($\Sigma C_t/C_\infty$) through membrane impregnated with phosphatidylcholine and removal of the phospholipid into the receiver phase ($\Sigma[P]_t/[P]_m$). The feed phase was in (A) a 0.3 mg/mL Zn^{2+}–insulin solution in deionized water (pH 3.0), in (B) a gel (PEG 8000, [NCO]/[OH] = 0.78) loaded with 0.2 wt % Zn^{2+}–insulin and swollen in ethanol, in (C) a gel (PEG 20,000, [NCO]/[OH] = 0.78) loaded with 0.2 wt % Zn^{2+}–insulin and swollen in (○, □, △) ethanol or in (●, ▲) aqueous solution, pH 3.0, and in (D) a gel (PEG 20,000, [NCO]/[OH] = 3.4) loaded with 0.2 wt % Zn^{2+}–insulin and swollen in ethanol. Initial receiver phase consisted of deionized water (pH 3.0) and was stirred in all experiments. Feed phase was stirred only in (A). Triangles designate $\Sigma[P]_t/[P]_m$, and squares and circles designate $\Sigma C_t/C_\infty$ for ethanol and insulin, respectively.

5(B) and (C)]. Notably, the release of small ethanol molecules was much less dependent on the gel parameters.

Does the protein released from the PEG gel and then transported through the skin-mimicking membrane into the aqueous solution retain its native structure? Far-UV CD spectra of the original insulin and the one released into the aqueous phase in the transport experiment are virtually identical (Fig. 6), thereby indicating the same α-helix content in the two species.[13,20]

While near-UV CD spectra, because of the low intensity, require a high concentration of protein, which is difficult to attain in a transport experiment, literature data on near-UV[13] suggest that insulin complexed with SDS retains its native structure even in hydrophobic solvent, such as 1-octanol. Similarly, Paradkar and Dordick[21] reported no spectroscopic differences for chymotrypsinogen ion paired with sodium bis(2-ethylhexyl)sulfosuccinate (Aerosol OT) and dissolved in isooctane or in aqueous buffer. Thus, evidence exists that proteins

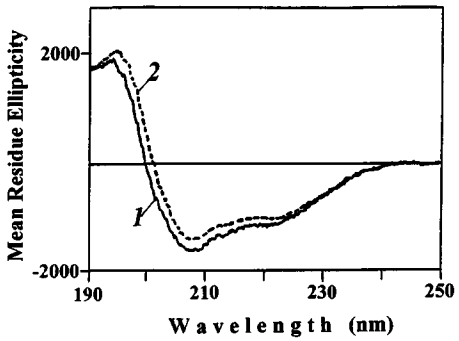


Figure 6 Far-UV CD spectra of (1) bovine Zn^{2+}–insulin in water at pH 3.0 and (2) insulin complexed with SDS, dissolved in ethanol, loaded into PEG gel (MW 20,000, [NCO]/[OH] = 1.0), and transported into aqueous receiver solution (pH 3.0) through phosphatidylcholine-impregnated membrane. The insulin concentration for both solutions was 8.5 μM.

dissolved in organic solvents via complexation with detergent are stabilized. Consequently, they remain in their native state when redissolved in an aqueous medium. This is further supported by the electronic absorption spectrum of ferric myoglobin transferred through the membrane from the PEG gel into an aqueous receiver solution (Fig. 7). The maxima of the Soret band (407 nm), as well as β and α bands due to the $\pi \rightarrow \pi^*$ electronic transitions in heme (505 and 637 nm, respectively), correspond to the native state of oxidized horse myoglobin.[22] Shifting to shorter wavelengths in ethanol (corresponding maxima are at 399, 500, and 621 nm) is caused by the interactions in the nondissociated complex of myoglobin and ligand (SDS).

In accordance with the foregoing concept, the enzymatic activity of lysozyme transported from 0.2

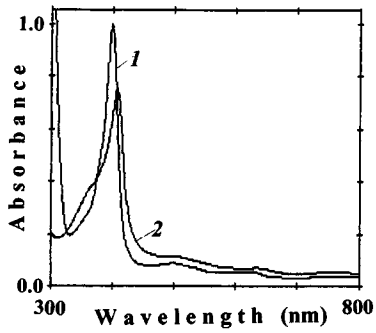


Figure 7 Absorbance spectra of (1) ethanolic solution of myoglobin/SDS (1 : 15 mol/mol) complex and (2) myoglobin complexed with SDS, dissolved in ethanol, loaded into PEG gel (MW 8000, [NCO]/[OH] = 1.0), and transported into aqueous receiver solution (pH 5.0) through the phosphatidylcholine-impregnated membrane.

wt % lysozyme–SDS in PEG gel (MW 20,000, [NCO]/[OH] = 0.86) through a phosphatidylcholine-impregnated membrane and collected in the receiver PBS phase for 2 days was measured to be the same, within experimental error, as the activity of the original lysozyme. After withdrawal, the receiver solutions were stored at 4°C prior to measurement of enzymatic activity (for the methods, see Experimental).

CONCLUSIONS

The formation of poly(ethylene glycol) networks crosslinked by trifunctional isocyanate has been described. Swelling of the networks in PBS and ethanol is governed by parameters of the initial mixture of PEG and isocyanate, such as the molecular weight of PEG and the ratio of equivalents of isocyano and hydroxyl groups. Protein loading into the gels from ethanol is enhanced by formation of hydrophobic complexes of ion-paired proteins and sodium dodecyl sulfate. Protein and ethanol release from PEG gels through phospholipid-impregnated membranes mimics that from the biphasic transdermal systems. Spectroscopic data and retention of enzymatic activity of the released proteins indicate that they remain in their native state upon release.

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