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Poly(L-lysine) as a model drug macromolecule with which to investigate secondary structure and microporous membrane transport, part 2: diffusion studies

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Abstract

Peptide drugs are hydrophilic in nature and so their preferred pathway of membrane transport is by the paracellular route, which primarily involves passive diffusion across intercellular pores. The objective of the present study was to investigate the effect of secondary structure on the aqueous diffusion of a model polypeptide, poly(L-lysine), through a microporous membrane. The primary aim was to systematically evaluate the variables (e.g. viscosity and/or hydrodynamic radius) that may contribute to the difference, if any, in the calculated values of the aqueous diffusion coefficient (D_{aq}) for each conformer of poly(L-lysine). Variations in pH and temperature of the medium were used to induce secondary structural changes in poly(L-lysine). Transport studies were conducted for 3 h at 25 or 37°C using side-by-side diffusion cells. Hydrophilic microporous polyester membranes with a 1-μm pore diameter were used to measure the free diffusion of each conformer. The values for the apparent permeability (P_{app}) and D_{aq} were calculated using standard equations. The viscosity of each conformer solution was determined and the hydrodynamic radius of each conformer was then estimated. At 25°C, both P_{app} and D_{aq} of the α-helix conformer were approximately the same as those of the random coil conformer. In contrast, at 37°C, the P_{app} and the D_{aq} of the β-sheet conformer were significantly ($P < 0.05$) less than those of the random coil conformer. At 25°C, the solutions containing primarily either the random coil or the α-helix conformers had approximately the same viscosity. On the other hand, at 37°C, the solutions containing the β-sheet conformer had a significantly ($P < 0.05$) higher viscosity than when this conformer was absent. The random coil and the α-helix conformers appeared to have comparable sizes, whereas the hydrodynamic radius estimated for the β-sheet conformer was significantly ($P < 0.05$) larger than those for the other two conformers. In summary, changing the secondary structure of poly(L-lysine) from the random coil to the α-helix did not affect its P_{app} and intrinsic D_{aq}. On the other hand, appearance of the β-sheet conformer significantly decreased the values of P_{app} and D_{aq}. The differences appeared to result from the significantly higher solution viscosity as well as the extended structure associated with the β-sheet conformer of poly(L-lysine). This strategy may represent a potential mechanism to sustain the delivery of therapeutic peptide drugs from a controlled drug delivery device.

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Introduction

Optimal delivery of therapeutic peptides and proteins still remains a significant challenge for pharmaceutical scientists. Among all potential routes of non-invasive drug administration, oral administration is the preferred route for long-term drug administration. In addition to the short post-absorptive residence time of these compounds in the systemic circulation, major barriers to drug delivery include poor membrane permeability and enzymatic stability of this class of therapeutic compounds (Lee & Robinson 2000).

Degradation of peptides and proteins can occur at the site of administration, in blood, liver, kidney, and during passage through vascular endothelium. There have been

attempts to overcome this problem by using enzyme inhibitors (Bernkop-Schnürch 1998) or by adding a pharmacologically inactive enzyme substrate. Mechanical protection by dosage forms such as microspheres or nanospheres have also been used (Kompella & Lee 2001). Another approach attempted is the modification of the peptide backbone by including D-isomers at the site of degradation to render the polypeptide an unnatural substrate for proteolytic enzymes (Pauletti et al 1997a).

Regardless of the route of administration, a drug has to pass through a number of biological barriers to exert its pharmacological effect. Poor membrane permeability is not unexpected for peptide and protein drugs since these compounds are large in molecular size as well as polar and charged in nature. Permeation enhancers have been employed in an effort to overcome this problem (Şenel & Hincal 2001). However, most of these agents are capable of perturbing membrane integrity resulting in possible damage to the mucosal surface with chronic use. Moreover, as a consequence of poor selectivity, uptake of immunogenic substances with associated systemic toxicity is a valid concern. Another approach that has been studied is chemical modification. Unfortunately, owing to the complex chemistry (both chemical and conformational) of peptides and proteins, rational approaches to changing the physicochemical properties without altering the biological activity are often not as readily achieved as with small organic molecules (Oliyai & Stella 1993).

Polypeptides and proteins in solution exhibit different secondary structures such as random coil, α-helix and β-sheet. These secondary structures exist in a dynamic equilibrium and the percentages of each conformer largely depend on the solution microenvironment. In addition, different secondary structures vary in physicochemical properties such as hydrophobicity, size and shape (Gray et al 1994). Poly(L-lysine), a synthetic homopolymer, was chosen as a model polypeptide in this study because of its limited tertiary structure in order to avoid confounding effects of tertiary structural changes. Variations in pH and temperature could be employed as stimuli to successfully induce a secondary structural change in poly(L-lysine) (Greenfield & Fasman 1969; Chittchang et al 2002).

Preliminary studies in our laboratory have demonstrated that the α-helix and the β-sheet conformers of poly(L-lysine) are significantly ($P < 0.05$) more lipophilic than the random coil, its native conformer (Chittchang et al 2002). Nevertheless, neither of the conformers is lipophilic enough to fall within the log K range of 1.5–3.5 necessary for maximal membrane permeation by the transcellular pathway (Lipinski et al 2001). This finding suggests that an increase in the hydrophobicity induced by changes in the secondary structure of a polypeptide is not likely to enhance its transcellular diffusion by increased partitioning into a lipid bilayer membrane.

Size and shape of each conformer tend to be different, for example an α-helix secondary structure is believed to be more compact than a random coil conformer (Lin & Dass 2001). A review of the literature reveals that the paracellular pathway is the primary route of mucosal membrane transport for polypeptides. In order to diffuse freely across the pores, the diameter of the molecule must be smaller than the pore size. The probability of a long, thin molecule oriented parallel to the cylindrical pore is low unless there is also bulk solvent flow. When the macromolecule becomes more compact with a smaller hydrodynamic radius, it may potentially pass through the pores more readily.

Paracellular transport primarily involves passive diffusion. The influence of molecular size and ionic charge on paracellular permeability has been extensively investigated (Pauletti et al 1997b). However, only small peptides ($\leqslant 6$ amino acids), in which the formation of secondary structures was not possible, were evaluated. Therefore, the objectives of the present study were to investigate the effect of secondary structure on the aqueous diffusion of a model polypeptide, poly(L-lysine), through a microporous membrane and to systematically evaluate the contributions of various diffusion parameters, such as viscosity of the medium and hydrodynamic radius of the permeant, to the difference, if any, in the calculated values of the aqueous diffusion coefficient (D_{aq}) for each conformer of poly(L-lysine).

Materials and Methods

Materials

Poly(L-lysine) HCl, molecular weight range 15000–30000 Da (with an average molecular weight of 28500 Da as determined by viscosity measurements), potassium phosphate (monobasic, anhydrous), boric acid (anhydrous), potassium chloride (anhydrous) and sodium hydroxide were purchased from Sigma Chemical Company (St Louis, MO, USA). Nuclepore Track-Etch membranes (polyester, 13 mm membrane diameter, 1 μm pore diameter) were obtained from Fisher Scientific (St Louis, MO, USA). Micro BCA reagents were purchased from Pierce (Rockford, IL, USA). All materials were used as received.

Induction of secondary structural changes

Variations in pH and temperature of the medium were used to induce secondary structural changes in poly(L-lysine) as reported previously (Figure 1) (Chittchang et al 2002). Phosphate buffer (10 mM) was used as the medium for pH 7.4, whereas alkaline borate buffer (10 mM) was used to maintain pH 9.0, 10.0 and 11.0. Before the addition of poly(L-lysine), the pH of both phosphate and alkaline borate buffer solutions were adjusted to the desired value. A sufficient amount of poly(L-lysine) HCl was dissolved to achieve a concentration of 1 mg mL^{-1} in the corresponding buffer. Following complete dissolution of poly(L-lysine), the pH of the solutions were determined and readjusted to desired values, if required. Solution pH was varied from 7.4 to 11.0 to attain various percentages of the random coil and the α-helix conformers. Two different percentages of the β-sheet conformer were obtained by heating the solutions of

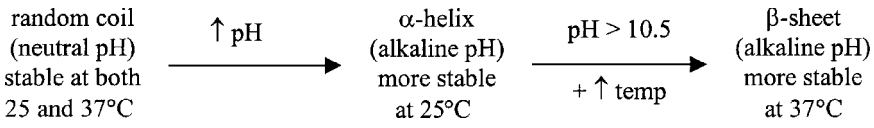

Figure 1 Induction of secondary structural changes in poly(L-lysine).

poly(L-lysine) in alkaline borate buffer (pH 11.0) to 50 or 55°C for 30 min. Resulting conformations were verified by circular dichroism (CD) studies.

All CD measurements were conducted using a Jasco J-720 spectropolarimeter (Japan Spectroscopic Co., Ltd, Tokyo, Japan) and a quartz cell with a volume of 150 μL and a 0.2-mm light path length. Each sample measurement was performed in triplicate scans and the average ellipticity values were automatically reported in millidegrees. The spectra thus obtained were analysed in the region of 190–250 nm to determine the percentages of each conformer present in the solutions with the JFIT program (developed by Bernhard Rupp, Lawrence Livermore National Laboratory, Livermore, CA, USA; www-structure.llnl.gov/cd/cdtutorial.htm).

Previous results from our laboratory indicated that experiments involving the heat-induced β-sheet conformer of poly(L-lysine) need to be conducted at a temperature greater than, or equal to, 37°C (Chittchang et al 2002). On the other hand, the α-helix conformer must be investigated at a temperature lower than, or equal to, 25°C, while the random coil conformation can be studied at either 25°C or 37°C, hence serving as a control for all experiments (Chittchang et al 2002). To parallel the results of our conformer-retention studies (Chittchang et al 2002), the random coil and the α-helix conformers were studied at 25°C, while the β-sheet conformer was compared with the random coil conformer at 37°C in all studies, unless otherwise stated.

Transport studies

Transport studies were conducted under sink conditions across the microporous membrane for 3 h at either 25 or 37°C using side-by-side diffusion cells (Crown Glass Company, Inc., Somerville, NJ, USA) with a donor concentration of 1 mg mL^{-1}. A 0.64-cm^2 area of the membrane was exposed to the continuously stirred donor and receptor compartments, each containing 3-mL fluid.

Solutions of each conformer were prepared as described above and placed in the donor chamber, while the corresponding buffer (same pH as the donor solution) was simultaneously added to the receptor side. Samples (200 μL) were obtained from the receiver compartment at pre-determined time points (0, 5, 10, 15, 30, 45, 60, 90, 120, 150 and 180 min) with buffer replacement. At the end of each experiment, CD measurements were performed on aliquots of the donor and receptor solutions in order to verify that the desired percentages of each conformer were maintained throughout the diffusion experiments.

The samples were analysed for poly(L-lysine) in duplicate using the Micro BCA Protein Assay method modified for 96-well microtiter plates. Briefly, 100 μL of working reagent was added to a 100-μL aliquot of the samples in the wells of the microtiter plate. Following thorough mixing and incubation for 1 h at 60°C, the absorbance of the samples were determined at 562 nm using a Bio-Rad Model 450 microplate reader (Bio-Rad Laboratories, Richmond, CA, USA). Since the reactivity of each conformer with the Micro BCA working reagent may differ, separate calibration curves were constructed for each conformer using the standard solutions prepared under similar conditions to those for the samples. The linear range of the calibration curve relating blank-corrected absorbances to poly(L-lysine) concentrations was subsequently used to determine the amount of poly(L-lysine) transported to the receptor compartment by diffusion across the microporous membrane. The dilution caused by buffer replacements was taken into account when determining the amount of poly (L-lysine) transported and mass balance calculations were also performed.

The apparent permeability (P_{app} in cm s^{-1}) and D_{aq} (in cm^2 s^{-1}) through the microporous membrane were estimated using equations 1 (Hidalgo 1996) and 2 (Pade & Stavchansky 1997).

$$P_{app} = (\Delta Q/\Delta t)/(A \times C_0) \quad (1)$$

$$D_{aq} = (P_{app} \times l)/(\epsilon \times F(r/R)) \quad (2)$$

where $\Delta Q/\Delta t$ is the slope of the cumulative amount diffused into the receptor compartment vs time plot (μg s^{-1}), A is the surface area of the exposed membrane (0.64 cm^2), C_0 is the donor concentration at time zero (1000 μg mL^{-1}), l is the membrane thickness (11 μm), ϵ is the porosity of the membrane (0.079, calculated from the total cross-sectional area of all pores per 1-cm^2 membrane area), and F(r/R) is the dimensionless Renkin molecular sieving function (($\cong$ 1 as the radius of the pore (R) is much larger than the radius of the molecule (r)). Since the membrane pores were uncharged, the electrochemical energy function term was not included in equation 2.

Viscosity measurements

Solutions of each conformer were prepared as described above. The density of the sample and buffer solutions were determined using a pycnometer with a capacity of 10 mL. The viscosity of each solution was then determined using a calibrated glass capillary viscometer (Cannon-Fenske routine type for transparent liquids, ASTM size 50, minimum

sample volume = 7 mL; Cannon Instrument Company, State College, PA, USA). Average flow time from five measurements, as well as the density determined at a corresponding temperature, were incorporated in the calculation of the viscosity (η in mPa s or cP) (equation 3).

$$\eta = k \times \rho \times t \quad (3)$$

where k is the viscometer constant ($mm^2\ s^{-2}$) at the corresponding temperature determined from the measured flow time and the published values of the viscosity and density of water (Lide 2000), ρ is the density of the solution ($g\ mL^{-1}$), and t is the average flow time (s). Moreover, the relative viscosity of the sample solution compared with buffer ($\eta_{sample}/\eta_{buffer}$) was calculated.

The Stokes-Einstein equation (equation 4) was used to estimate the hydrodynamic radius (r_H in cm) from the estimated D_{aq} and the viscosity of each conformer solution.

$$r_H = (k \times T)/(6 \times \pi \times \eta \times D_{aq}) \quad (4)$$

where k is the Boltzmann constant, T is the temperature (K), η is the viscosity (P), and D_{aq} is the aqueous diffusion coefficient ($cm^2\ s^{-1}$).

The Renkin molecular sieving function, F(r/R), for flexible polymers through cylindrical channels was also calculated using the estimated hydrodynamic radius of each conformer as the molecular radius in equation 5 (Davidson & Deen 1988).

$$F(r/R) = [1-(r/R)]^2 \times [1 - 2.848(r/R) + 3.269(r/R)^2 - 1.361(r/R)^3] \quad (5)$$

where r is the molecular radius (Å) and R is the pore radius (5000 Å).

Finally, the frictional ratio (F) was calculated using the following equations (Tanford 1961; Munk 1989).

$$F = f_{exp}/f_0 = (kT/D_{aq})/(6 \times \pi \times \eta \times r_s) \quad (6)$$

$$r_s = [(3 \times MW \times \bar{\nu})/(4 \times \pi \times N_A)]^{1/3} \quad (7)$$

in which k is the Boltzmann constant, T is the absolute temperature (K), D_{aq} is the aqueous diffusion coefficient ($cm^2\ s^{-1}$) obtained from the microporous membrane diffusion experiments, η is the viscosity (P), r_s is the radius (cm) defined for a sphere, MW is molecular weight ($g\ mol^{-1}$), $\bar{\nu}$ is the partial specific volume of the polypeptide ($cm^3\ g^{-1}$), and N_A is Avogadro's number.

Statistical analysis

All experiments were performed in triplicate, unless otherwise stated, and the results are expressed as the mean value ± s.d. Differences among group means were determined by either the two-tailed Student's *t*-test or a one-way analysis of variance with multiple range tests using the Fisher's least significant difference procedure. The differences were deemed significant at $P < 0.05$.

Results and Discussion

Induction of secondary structural changes

At neutral pH, the ϵ-amino groups of poly(L-lysine) are positively charged and a major proportion of this polypeptide exists as the random coil conformer. The effect of pH on the percentages of each conformer present in a poly(L-lysine) solution is shown in Figure 2. At all pH values, only two conformers existed in the solutions, that is the random coil and the α-helix conformers. When the pH was raised, the lysyl side chains were neutralized and the α-helix structure was consequently formed by intramolecular hydrogen bonding. Interestingly, when the data points were interpolated, the two lines intersected and the ratio of these two conformers became 50:50 at a pH of approximately 10.3–10.4, which is in good agreement with the reported pK_a (10.0–10.3) of the lysyl side chains (Hermans 1966; Pederson et al 1971). This result confirms the mechanism of charge neutralization for the induction of the α-helix conformer of poly(L-lysine). When the pH equals the pK_a of the lysyl side chains, 50% of the molecules are neutralized and adopt the α-helix structure, while the remaining 50% exist as the random coil conformer.

The minimum temperature required for induction of the β-sheet conformer has been reported to be 50°C (Fasman 1989). From our previous studies, a further increase in temperature to 60°C did not result in a higher percentage of the β-sheet conformer than when 55°C was used (Chittchang et al 2002). Therefore, two temperatures, 50 and 55°C, were used to induce the β-sheet structure in the present study. Percentages of each conformer in the solutions are listed in Table 1. The thermal energy desolvates the molecules such that hydrogen bonding between neighbouring chains may occur, resulting in the formation of the β-sheet conformer. The proposed mech-

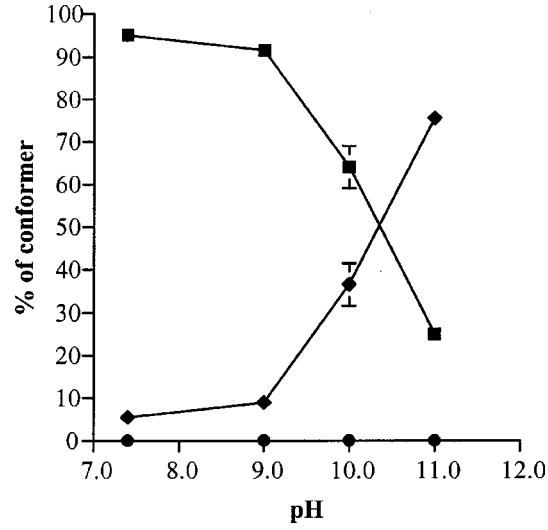

Figure 2 Effect of pH on the percentage of each conformer in poly(L-lysine) solution at 25°C: random coil (■), α-helix (◆), and β-sheet (●). Data points represent the mean ± s.d. of three experiments. Lines through the mean values do not represent a mathematical fit.

Table 1 Effect of heating temperature on the percentage of each conformer in the solution of poly(L-lysine) in alkaline borate buffer (pH 11.0).

Conditions	Random coil (%)	α-Helix (%)	β-Sheet (%)
pH 7.4 (control)	94.77±0.95	5.24±0.95	0.00±0.00
pH 11.0, heated to 50°C for 30 min	11.10±1.45	36.90±4.02	52.00±5.26
pH 11.0, heated to 55°C for 30 min	9.21±3.45	17.23±2.90	73.57±1.88

All data represent the mean value±s.d. of three experiments.

anism is supported by the evidence that the surfaces of the α-helix conformation of poly(L-lysine) are hydrated by four to six molecules of water per lysine residue, whereas the surfaces of the β-sheet conformer have less than two molecules of water per lysine residue (Blout & Lenormant 1957).

Transport studies

Membranes with large pores (i.e. membranes in which the ratio of the effective molecular radius to the pore radius is less than 0.01) have been used for measurements of the diffusion coefficient in bulk solution (Deen et al 1981). When the average pore radius of these membranes is much larger than the Stokes-Einstein radius of the solutes, steric and hydrodynamic hindrances to diffusion become negligible. As a result, D_{aq} approaches the value in the absence of constraints introduced by the pores (D_{∞}). Hindrance by the pores has been estimated by the Renkin molecular sieving function, which represents the ratio of the diffusion coefficients through the pores to that in the bulk solution (D_{aq}/D_{∞}) (Renkin 1954). Values close to unity imply no hindrance to diffusion by the pores.

It has been reported that the horseradish peroxidase with a molecular weight of 40 000 Da has a molecular diameter of about 50 Å (i.e. a radius of 25 Å) (Clementi & Palade 1969). When this molecular radius is used to calculate the Renkin molecular sieving function imposed by the pores with a 5000-Å radius (i.e. the ratio of the two radii is 0.005), a value of 0.98 is obtained. Such a value close to unity suggests that the molecular size restriction imposed by the membranes used in this study should be negligible for molecules of approximately this size, including the model polypeptide with an average molecular weight of 28 500 Da.

CD studies indicated that the percentages of each conformer in both the donor and the receptor cells remained relatively constant throughout the 3-h experiments under the conditions employed in the present study (data not shown). This result is in good agreement with the stability study data reported previously by our laboratory (Chittchang et al 2002). Figure 3 shows the transport profile of poly(L-lysine) at 25°C at four pH values in which different percentages of the random coil and the α-helix conformers were present (as shown in Figure 2). Figure 4 is a plot of the calculated transport parameters (associated with Figure 3) as a function of the percentage of the α-helix conformer present. It is apparent from Figures 3 and 4 that an increase in the percentage of the α-helix conformer as a result of increasing pH did not significantly affect either the rate or the extent of poly(L-lysine) transport.

Analogous studies were conducted to compare the transport of the β-sheet structure with that of the random coil conformer at 37°C. The results are presented in Figures 5 and 6. Despite only 52% of the poly(L-lysine) molecules existing as the β-sheet structure after the solution was heated to 50°C for 30 min, the amount of poly(L-lysine) transported across the microporous membrane at all time points, as well as other transport parameters, were significantly ($P < 0.05$) lower than in the absence of the β-sheet conformer (i.e. at pH 7.4). An increase in the percentage of the β-sheet structure to 74%, induced by heating the solution to 55°C for 30 min, did not result in a significant ($P > 0.05$) decrease in the transport parameters when compared with the corresponding values for the solution heated to 50°C for 30 min.

A 500 MHz model DRX500 Bruker Avance NMR equipped with Pulse Field Gradient Spin Echo software

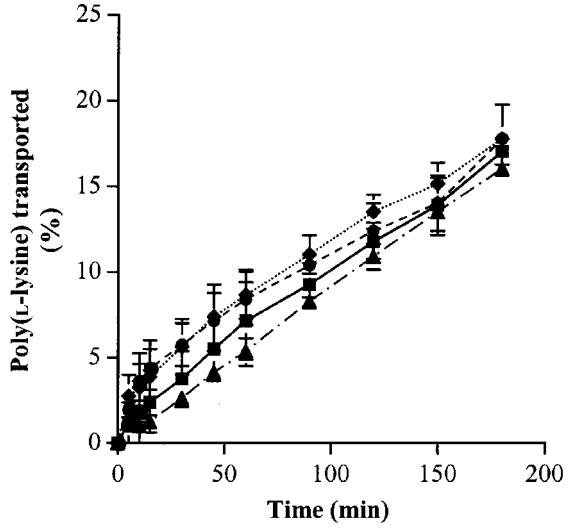

Figure 3 Percentage of poly(L-lysine) transported across a Nuclepore membrane with an average pore diameter of 1 μm at 25°C: pH 7.4 (■), pH 9.0 (◆), pH 10.0 (●) and pH 11.0 (▲). Data points represent the mean±s.d. of three experiments. Lines through the mean values do not represent a mathematical fit.

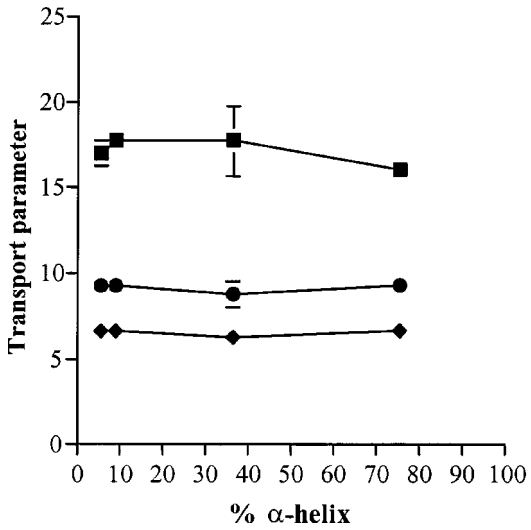

Figure 4 Effect of the percentage of the α-helix conformer on the transport parameters of poly(L-lysine) at 25°C: % transported (■), apparent permeability $\times 10^5$ (in cm s^{-1}) (◆), and aqueous diffusion coefficient $\times 10^7$ (in cm^2 s^{-1}) (●). Data points represent the mean ± s.d. of three experiments. Lines through the mean values do not represent a mathematical fit.

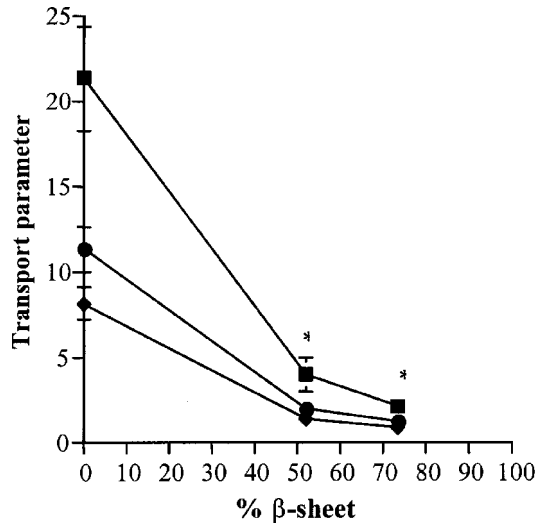

Figure 6 Effect of the percentage of the β-sheet conformer on the transport parameters of poly(L-lysine) at 37°C: % transported (■), apparent permeability $\times 10^5$ (in cm s^{-1}) (◆), and aqueous diffusion coefficient $\times 10^7$ (in cm^2 s^{-1}) (●). Data points represent the mean ± s.d. of three experiments. Lines through the mean values do not represent a mathematical fit. $^*P < 0.05$, significant reduction in transport parameters (associated with Figure 5) for both solutions containing the β-sheet conformer compared with the solution containing primarily the random coil secondary structure.

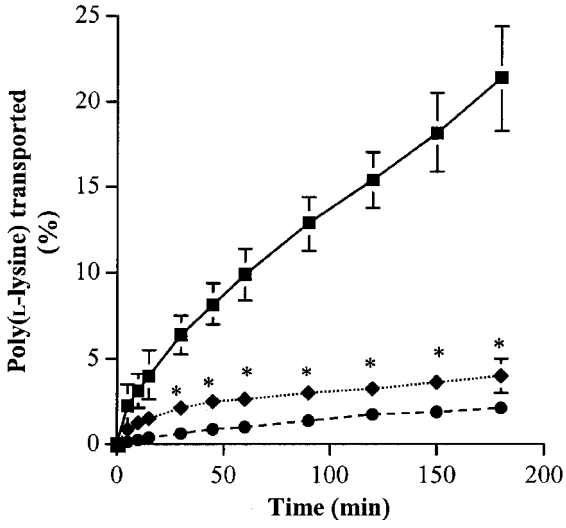

Figure 5 Percentage of poly(L-lysine) transported across a Nuclepore membrane with an average pore diameter of 1 μm at 37°C: pH 7.4 (control) (■), pH 11.0 heated to 50°C (◆), and pH 11.0 heated to 55°C (●). The solutions were initially heated to the specified temperature for 30 min and then maintained at 37°C throughout the experiment. Data points represent the mean ± s.d. of three experiments. Lines through the mean values do not represent a mathematical fit. $^*P < 0.05$, significant reduction in the cumulative percentage of poly(L-lysine) transported at all time points for both solutions containing the β-sheet conformer compared with the solution containing primarily the random coil secondary structure.

was utilized to obtain a second estimation of the diffusion coefficients for each conformer. The NMR-estimated D_{aq} values were in close agreement with the values obtained from the diffusion studies (data not shown). This finding, together with a Renkin molecular sieving function of close to unity, further substantiates the suggestion that the microporous membranes used in this study did not hinder the diffusion of any conformer.

One could possibly argue that the difference in the transported amount of the random coil and the β-sheet conformers might result from the difference in charge between the positively charged random coil and the neutral β-sheet conformers. However, this difference also existed in the case of the random coil and the α-helix conformers, which were transported to the same extent at 25°C. Moreover, the polyester membranes used in this study were neutral, unlike the situation in-vivo in which tight junctional complexes are negatively charged (Rojanasakul et al 1992; Gonzáles-Mariscal et al 2001). Therefore, charge is not likely to play a role with regard to polypeptide diffusion in this case. Other potential reasons, for example solution viscosity and hydrodynamic radius, need further examination.

Viscosity measurements

From the Stokes-Einstein equation, the diffusion coefficient at a specific temperature is influenced by the molecular radius and the viscosity of the medium. Viscosities of all the buffer solutions used in the present study were comparable with the viscosity of water at the same temperature (data not shown). However, it has been observed that polymers present in solutions tend to influence their viscosity (Munk 1989).

Table 2 Viscosity of poly(L-lysine) solutions.

Temperature	Conditions (major conformer)	Viscosity (cP)	Relative viscosity (compared with buffer)
25°C	pH 7.4 (95% random coil)	0.89±0.02	1.02±0.02
	pH 11.0 (75% α-helix)	0.86±0.01	1.01±0.01
37°C	pH 7.4 (95% random coil)	0.73±0.01	1.05±0.01
	pH 11.0 heated to 50°C[a] (52% β-sheet)	0.99±0.07*	1.41±0.10*
	pH 11.0 heated to 55°C[a] (74% β-sheet)	1.02±0.02*	1.44±0.02*

All data represent the mean value±s.d. of three experiments. [a]The solutions were initially heated to 50 or 55°C for 30 min and then maintained at 37°C throughout the experiment. *$P < 0.05$, significant difference compared with the solution containing primarily the random coil conformer at 37°C (one-way analysis of variance with multiple range tests using the Fisher's least significant difference procedure).

Viscosities of selected poly(L-lysine) solutions containing one major conformer are listed in Table 2. At 25°C, the solutions containing either 95% random coil or 75% α-helix had approximately the same viscosity. Since the relative viscosities of both the solutions were practically unity, these two conformers clearly did not affect the viscosity of their solution. At 37°C, the viscosity of both buffer solutions (data not shown), as well as the solution containing primarily the random coil conformer, significantly decreased as expected at an elevated temperature (compared with 25°C). When either 52% or 74% of the molecules adopted the β-sheet structure, the solution viscosity increased to approximately the same extent ($P > 0.05$), which is almost 50% over that of the buffer. This significantly ($P < 0.05$) higher viscosity could be one of the reasons why the β-sheet conformer had a much lower D_{aq} compared with that of the random coil conformer.

Despite the fact that the Stokes-Einstein equation was first developed for small, spherical molecules, it has been found to accurately predict the diffusivity of large molecules such as globular proteins (30 Å radius) and latex (450 Å) (Dubin et al 1967; Conlon & Craven 1972). In the case of particles with irregular shape, a hydrodynamic radius (r_H) is assigned as an estimate of their size (Munk 1989). Even though this parameter has no real physical meaning, it provides some information about the radius of a hypothetical sphere that has the same frictional coefficient, and hence the same diffusion coefficient, in the same liquid as the actual particle.

The hydrodynamic radius of the major conformer in the solution was estimated using the Stokes-Einstein equation (equation 4) with the experimentally determined viscosity for each solution. In order to verify the assumption of negligible hindrance imposed by the membrane pores, the Renkin molecular sieving function was subsequently calculated from the estimated hydrodynamic radius. As shown in Table 3, the random coil and the α-helix conformers appeared to have comparable sizes. Moreover, based on the calculated values for the molecular sieving function, the diffusion of these two conformers was not likely to be hindered by the microporous membranes. Approximately the same hydrodynamic radii of the random coil conformer obtained at 25 and 37°C indicate that the greater D_{aq} at 37°C resulted primarily from the decrease in viscosity at this higher temperature. In the case of the β-sheet conformer, even after the enhanced viscosity was taken into consideration, its estimated hydrodynamic radius was still five to six times greater than the other two conformers ($P < 0.05$). It can be clearly seen from Table 3 that the estimated hydrodynamic radius increased significantly ($P < 0.05$) as the percentage of the β-sheet conformer increased from 52 to 74%. The only explanation for the same molecule exhibiting such a large difference in hydrodynamic radii is the adoption of two significantly different shapes. Such an assumption appears to be reasonable in view of the fact that the β-sheet conformer is known to be an extended structure. The extended structure might be the reason why this conformer experiences more hindrance by the pores as reflected by a significantly ($P < 0.05$) smaller value of the Renkin molecular sieving function (Table 3).

The frictional ratio (F or f_{exp}/f_0) is defined as the ratio of the apparent frictional coefficient of a molecule to that of a hypothetical sphere of identical volume (Tanford 1961). It represents the deviation of the protein hydrodynamic particle from the shape of a sphere. Values of the frictional ratio over the range of 1.25–1.40 indicate minimal deviation from a spherical shape, as was the case with solutions in which the random coil or the α-helix was the predominant conformer. On the other hand, when the β-sheet conformer was present in the solutions, the frictional ratio substantially deviated from unity, suggesting a highly elongated prolate ellipsoid (Schürmann et al 2001) experiencing a

Table 3 Estimated hydrodynamic radius, Renkin molecular sieving function, and frictional ratio of poly(L-lysine).

Temperature	Conditions (major conformer)	Estimated hydrodynamic radius (Å)	Renkin molecular sieving function	Frictional ratio
25°C	pH 7.4 (95% random coil)	27.0±1.1	0.974±0.001	1.3±0.1
	pH 11.0 (75% α-helix)	27.8±1.7	0.973±0.002	1.3±0.1
37°C	pH 7.4 (95% random coil)	28.1±3.2	0.973±0.003	1.3±0.2
	pH 11.0 heated to 50°C[a] (52% β-sheet)	127±25*†	0.883±0.022*†	6.0±1.2*†
	pH 11.0 heated to 55°C[a] (74% β-sheet)	193±27*†	0.828±0.022*†	9.2±1.3*†

All data represent the mean value±s.d. of three experiments. [a]The solutions were initially heated to 50 or 55°C for 30 min and then maintained at 37°C throughout the experiment. *$P < 0.05$, significant difference compared with the solution containing primarily the random coil conformer at 37°C (one-way analysis of variance with multiple range tests using the Fisher's least significant difference procedure). †$P < 0.05$, significant difference between the denoted values in a given column.

much greater translational friction than a sphere of identical volume. Such higher frictional resistance could be reasonably expected for an extended β-sheet structure.

The results of the present study indicate that changing the secondary structure of a polypeptide to the β-sheet conformation is likely to affect its aqueous diffusion. This may potentially be a strategy to control the diffusion of a polypeptide through a rate-limiting membrane contained in a controlled drug delivery device. Polymeric membranes have been used to control the rate of drug release from delivery systems containing a drug reservoir that may exist in solid, suspension, or solution form (Heller 1987; Chien 1992). The membrane can be fabricated from a non-porous polymeric material or a microporous membrane. Drug release from this type of delivery device is normally controlled at a pre-programmed rate by controlling the partition coefficient and diffusivity of the drug molecule and the thickness of the rate-controlling membrane. Dosage forms of this type have an advantage of a zero-order release as long as the activity of the drug remains constant, i.e. for a solid phase or saturated solution load (Baker & Lonsdale 1974), which is usually not possible for expensive water-soluble peptide or protein drugs. However, this effect might be achieved by the induction of the β-sheet conformer in an unsaturated drug solution reservoir. Further studies are required to verify the reversibility of secondary structural changes back to the native conformer along with preservation of biological activity on a case-by-case basis for therapeutic peptides.

Polymeric membrane permeation-controlled drug delivery systems also offer the advantage of mechanically protecting the biologically active polypeptides from enzymatic degradation provided that the enzyme's size and associated shape do not permit its diffusion into the drug reservoir of the drug delivery device. Further improvement may be achieved by the development of delivery systems containing mucoadhesive polymers that provide intimate contact with the underlying mucosa, thus increasing the residence time and reducing the extent of drug degradation between the delivery system and the absorbing membrane by luminally secreted proteases (Bernkop-Schnürch 1998). Additionally, controlled-release drug delivery systems could be designed to provide simultaneous release of a drug and an enzyme inhibitor (Bernkop-Schnürch 1998).

Conclusions

Changing the secondary structure of poly(L-lysine) from the random coil to the α-helix did not affect its P_{app} and intrinsic D_{aq}. On the other hand, appearance of the β-sheet conformer significantly decreased the values of P_{app} and D_{aq}. The differences appeared to result from the significantly higher solution viscosity as well as the extended structure associated with the β-sheet conformer of poly(L-lysine). This strategy may represent a potential mechanism to sustain the delivery of therapeutic peptide drugs from a controlled drug delivery device employing a microporous rate-limiting membrane.

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