Prediction of the Viscosity Radius and the Size Exclusion Chromatography Behaviour of PEGylated Proteins

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Abstract

Size exclusion chromatography (SEC) was used to determine the viscosity radii of equivalent spheres for proteins covalently grafted with poly(ethylene glycol) (PEG). The viscosity radius of such PEGylated proteins was found to depend on the molecular weight of the native protein and the total weight of grafted PEG but not on PEG molecular weight, or PEG-to-protein molar grafting ratio. Results suggest grafted PEG's form a dynamic layer over the surface of proteins. The geometry of this layer results in a surface area to volume ratio approximately equal to that of a randomly coiled PEG molecule of equivalent total molecular weight. Two simple methods are given to predict the viscosity radius of PEGylated proteins. Both methods accurately predicted (3% absolute error) the viscosity radii of various PEG-proteins produced using three native proteins, α-lactalbumin (14.2 kDa MW), β–lactoglobulin dimer (37.4 kDa MW) and bovine serum albumin (66.7 kDa MW), three PEG reagents (2400, 5600, and 22500 MW), and molar grafting ratios of 0 to 8. Accurate viscosity radius prediction allows calculation of the distribution coefficient, K_{av}, for PEG-proteins in SEC. The suitability of a given SEC step for the analytical or preparative fractionation of different PEGylated protein mixtures may therefore be assessed mathematically. The methods and results offer insight to several factors related to the production, purification, and uses of PEGylated proteins.

Keywords: chromatography, gel filtration, PEG, protein, viscosity radius, size

Introduction

Covalent modification of proteins with poly(ethylene glycol) (PEG) molecules often dramatically improves their clinical efficacy. Terminal end functionalised PEG molecules are typically used to modify proteins by reacting with protein amine or other (e.g. sulfhydryl) reactive groups (*1, 2*). PEGylation may (a) mask and reduce immune-recognition and clearance from the body, (b) protect from enzymatic or chemical alteration, and clearance (*3-5*) (c) enhance hydrodynamic size and reduce glomerular filtration (*2*). The effectiveness of small therapeutic proteins (< 20 kDa) is particularly prone to enhancement through PEGylation (*6-8*). Many PEGylated therapeutic proteins are FDA approved and more are under development as PEGylation increases efficacy, reduces dosage frequency, allows for novel dosage mechanisms and may prolong shelf-life (*4, 5, 9*).

The major drawback of PEGylation is that PEGylated proteins are prepared from pure proteins, which are converted to product mixtures of PEG, native protein, and PEG-proteins having varied PEGylation extent (also known as degree of modification or grafting ratio, *N*) and variation in the site(s) of modification. Such PEGylated proteins may vary substantially in both physicochemical and biomedical properties. Low molecular weight by-products formed during PEGylation and/or hydrolysis of functionalised PEG's also add to product complexity.

The extent of PEGylation must routinely be determined in research, development and production laboratories. The current method of choice for analysis is mass spectrometry, particularly matrix-assisted laser desorption ionization - time of flight mass spectrometry (MALDI-TOF MS). Other methods include capillary electrophoresis (*10*) or light scattering (*11*). All such methods are relatively complex, require specialised equipment and have little direct relationship to the chromatographic, filtration and other methods used to purify PEGylation reaction product mixtures.

Size exclusion chromatography (SEC) and related methods are attractive for fractionation of native and PEGylated proteins due to the significant increases in protein viscosity radii which result from PEG grafting. In addition, PEGylation may variously alter protein properties and behaviour in regard to solubility, electrophoresis, ion exchange, affinity or other interactions typically used to facilitate their purification.

SEC is also a simple, low-cost technique that has been used for many decades to estimate the molecular weight (MW) of proteins and polymers through the use of calibration curves between molecular weight and the distribution coefficient,

$$K_{av} = \frac{(V_R - V_o)}{(V_c - V_o)} \qquad (1)$$

where V_R, V_o and V_c represent solute elution volume, void volume and the total bed volume of fluid and SEC media combined, respectively.

Given the above, SEC is attractive for both the purification and characterisation of PEG-proteins, and there is a significant need to be able to predict SEC results based only on native protein MW, PEG MW, and *N*. However proteins and PEG molecules of similar MW differ greatly with respect to their distribution coefficients in SEC media (*12*). This is illustrated by Figure 1 which shows calibration curves for protein and PEG molecular weight standards in a Superdex 200 HR10/30 SEC column (Amersham Biosciences, Uppsala, Sweden).[a]

[a] For ease of comparison, protein and polymer molecular weights determined by various methods are herein referred to in Daltons (Da) or kDa.

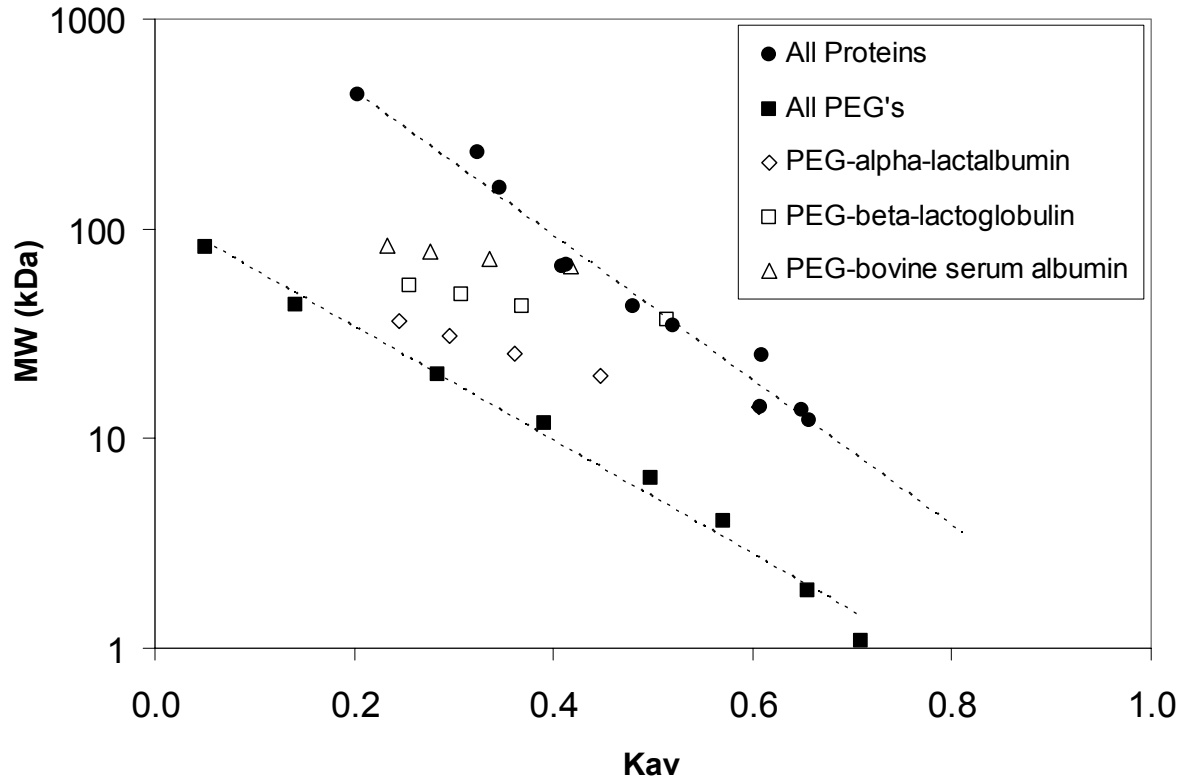

Figure 1. Distribution coefficient, K_{av}, as a function of $\ln(M_r)$ for protein and PEG molecular weight standards in a Superdex 200 HR 10/30 gel filtration column. Also shown are α-lactalbumin, β-lactoglobulin and BSA, PEGylated to various extents with mPEG-SPA 5000.

PEG-proteins are hybrid molecules and their SEC column distributions should not necessarily mimic that of either proteins or PEG molecules (*13*). One solution would be to calibrate SEC columns for PEG-proteins using protein MW standards modified with defined types of PEG's at defined degrees of modification. Clearly such calibrations are tedious, expensive, and may not pertain to PEG-proteins produced using novel PEG's or proteins. As such there is a significant need to develop simple methods to predict the SEC behaviour of PEG-proteins.

Such a need is supported by previous literature. M^{c}Goff et al. (*14*) fractionated samples of PEGylated superoxide dismutase by SEC and concluded that determination of PEG-protein

molecular weights based on protein standards was unreliable. Fortier and Laliberté (*15*) used protein MW standards and SEC to estimate the MW's of PEGylated horseradish peroxidase samples. They noted significant deviations from molecular weights estimated by other means, and that use of the SEC results to estimate *N* gave non-integer values, e. g. ranging from 3.1 to 6.0 against an expected value of 4 (lysines per protein). Christakopoulos et al. (*16*) cautioned about use of SEC to estimate PEG-protein MW's but nevertheless used SEC to relate an average increase of 30 to 40 kDa in the apparent MW of endogluconase to estimate *N* as 6 to 8 PEG molecules added per protein molecule. Veronese (*17*) noted that elution times of PEGylated proteins in SEC are not directly related to the increase in molecular weight due to the linked PEG, the implication being that SEC is not a useful analysis method for determining the degree of PEGylation. Fee (*13*) recently showed the apparent MW's of α-lactalbumin and β-lactoglobulin PEGylated with 5000 MW PEG increased non-linearly between 50 and 100 kDa per grafted PEG molecule, when estimated by SEC calibrated with protein MW standards. However the SEC MW estimates increased linearly by 7 kDa when the column was calibrated with PEG MW standards.

The present work addresses the need for a simple method to predictively model the SEC behaviour of PEGylated proteins. The viscosity radii of PEG-proteins were determined using several SEC columns and two models for predicting the viscosity radii (and therefore the distribution coefficients) of PEG-proteins were derived. The first approach is based on a proposed linear relationship between total grafted PEG MW and PEGylated protein size, leading to a simple predictive equation dependent on two experimentally determined constants, *a* and *b*. The second approach assumes that PEG-protein size is determined primarily by the conformation of the grafted PEG layer. The resultant model depends only on the viscosity radii of the native protein and PEG's, and contains no experimentally determined parameters.

The models were tested experimentally using PEG-protein samples prepared using three different proteins of MW 14 to 67 kDa, three different PEG reagents of MW 2 to 20 kDa and *N* values of 1 to 8. Both models provide an accurate prediction of the viscosity radii of PEG-proteins. Both enable the prediction of SEC to separate different PEG-proteins, and the identification of *N* by SEC using a single calibration curve, based on protein or PEG or a combination of protein and PEG molecular weight standards. In addition to their practical significance, the predictive accuracy of the models suggests they provide insight to the possible behaviour of PEG's when grafted to globular protein surfaces.

Experimental Methods

Apparatus and Materials

Chromatographic experiments were carried out on an AKTAFPLC™ liquid chromatography system with protein species detected by UV absorbance at 280nm (Amersham Biosciences, Uppsala, Sweden). PEG and PEG-containing species were detected with a Waters Model 410 Refractive Index detector (Waters Corporation, Milford, MA, USA). The chromatography system was operated using Unicorn™ software, version 4.1 (Amersham Biosciences, Uppsala, Sweden).

Superdex 200 HR 10/30 and Superdex 75 HR 10/30 pre-packed columns (30 cm length x 1.0 cm i.d., nominal d_p = 13 μm, V_c = 23.562 mL) were obtained from Amersham Biosciences (Uppsala, Sweden).

Phosphate buffered saline (PBS), Tris-HCl, Blue Dextran (M_r = 2000000), α-lactalbumin from bovine milk (85% pure by polyacrylamide gel electrophoresis (PAGE)), β-lactoglobulin from bovine milk (90% pure by PAGE) and bovine serum albumin (BSA) from bovine

plasma (98% pure by PAGE) were purchased from Sigma (St. Louis, MO, USA). The nominal molecular weights of α-lactalbumin, β-lactoglobulin dimer and BSA were, respectively, 14.2, 36.0 and 66.0 kDa and were estimated by MALDI-TOF mass spectrometry to be 14.2, 37.4 and 66.7 kDa, respectively. Mono-methoxy-poly(ethylene glycol)-succinimidyl propionate (mPEG-SPA) reagents of nominal molecular weights 2000, 5000 and 20000 Da were purchased from Nektar Therapeutics (Huntsville, AL, USA). The actual molecular weights were estimated by MALDI-TOF mass spectrometry to be 2.4, 5.6 and 22.5 kDa, respectively.

Low Molecular Weight and High Molecular Weight gel filtration calibration kits containing ribonuclease A (M_r 13.7 kDa), chymotrypsinogen A (M_r 25 kDa), ovalbumin (M_r 43 kDa), albumin (M_r 67 kDa), aldolase (M_r 158 kDa), catalase (M_r 232 kDa) and ferritin (M_r 440 kDa) were obtained from Amersham Biosciences (Uppsala, Sweden). Polyethylene Glycol 106 - 20000 MW, and Polyethylene Oxide 20000 - 1000000 MW Size Exclusion Calibration kits, containing molecular weights of 1100, 1900, 4000, 6500, 11800, 20400, 43500 and 82300, were purchased from Polymer Laboratories Ltd (Shropshire, UK).

Methods

Size-exclusion columns were calibrated against protein and PEG molecular weight standards. Individual solutions of each standard were prepared according to the manufacturer's instructions. 50 μL samples were injected onto each size exclusion column, using PBS running buffer at 0.5 mL/min. UV absorbance at 280 nm (protein standards) and refractive index (PEG standards) were used to determine the elution volumes of each standard. V_o was measured by the elution volume of Blue Dextran (M_r = 2000000) marker. V_o values were found to be 7.97 and 7.83 mL for the Superdex 200 and the Superdex 75

columns, respectively. K_{av} values were calculated for each column using equation (1) and plotted against molecular weight. Figure 1 shows the calibration curves for the Superdex 200 HR 10/30 column. Similar curves were obtained for the Superdex 75 column (data not shown).

SEC curves for individual samples of α-lactalbumin, β-lactoglobulin (dimer) and BSA proteins, and mPEG-SPA 5000 PEGylation reagent are plotted in Figure 2. All had retention volumes consistent with their native molecular weights according to corresponding MW calibration curves. Corresponding curves for mPEG-SPA 2000 and mPEG-SPA 20,000 were omitted for clarity but they exhibited peak maxima at retention volumes consistent with their molecular weights. The UV active peak eluting at about 20 mL is believed to represent N-hydroxysuccinimide acid by-product of the hydrolysis of mPEG-SPA.

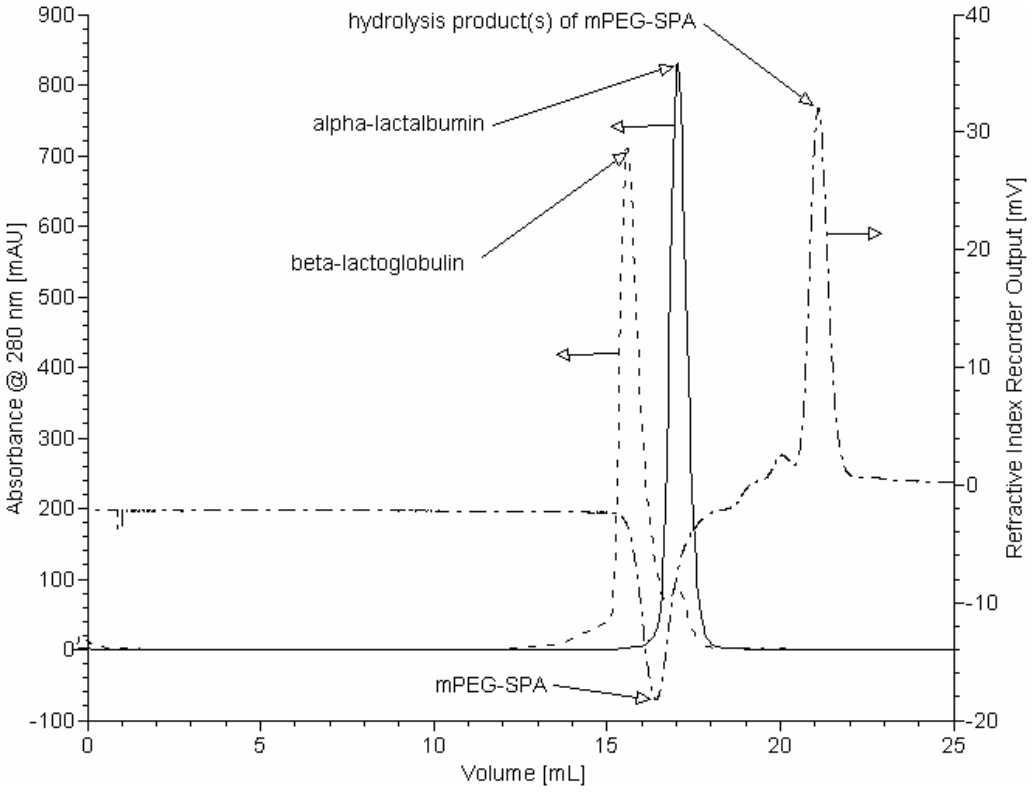

Figure 2. Chromatograms of native protein and mPEG-SPA 5000 reagent species in a Superdex 200 HR 10/30 SEC column.

Batch PEG grafting was carried out for each protein individually. All solutions were made up in PBS at pH 7.4. Typically 5 mL of 20 mg/mL mPEG-SPA solution was added to 5 mL of 20 mg/mL native protein and stirred in an open 10 mL beaker at room temperature. One sample (approx. 1 mL) was withdrawn immediately after mixing the reactants and further samples were taken at 2, 5, 10, 20, 30, 40, 50 and 60 minutes. Samples were immediately acidified with three drops of 0.1M HCl to stop the reaction. 50 μL of each sample was then analysed separately by SEC under the same conditions as used during column calibration. 100 μL samples were used when collecting fractions for MALDI-TOF analysis. In such cases fractions containing protein were collected in 1 mL samples and exchanged into 20 mM ammonium bicarbonate buffer using a HiTrap desalting column (5 mL) (Amersham Biosciences, Uppsala, Sweden) prior to MALDI-TOF analysis.

An Ettan II MALDI-TOF mass spectrometer (Amersham Biosciences, Uppsala, Sweden) operated in linear mode at 20 kV, was used to determine the molecular weights of native and PEGylated proteins. The matrix was prepared by saturating a solvent (consisting of 500 μL each of acetonitrile and MilliQ water and 9 μL trifluoroacetic acid) with sinapic acid. Equal volumes of sample and matrix solution were mixed and 0.3 μL of the mixture was applied to the sample plates.

Results and Discussion

Figure 3 shows a representative size exclusion chromatograph obtained in the Superdex 200 column for the products of batch PEGylation of α-lactalbumin by mPEG-SPA 5000. As expected, several products with distinctly different molecular sizes result from the modification of the protein and there is a shift in the areas of the product peaks with time as

the degree of PEGylation increases. Similar results were observed for batch PEGylation of β-lactoglobulin and BSA (data not shown for brevity). Such behaviour has been extensively reported in the literature (*5*). The reaction products were tentatively identified as mono-, di-, tri- and tetra-PEGylated protein by inference from progression of the reaction and the chromatograph in figure 3. Fractions were then collected in a repeat SEC run with a larger sample and the molecular weights were confirmed by MALDI-TOF. As noted in the previous section the peak at 20 mL may represent a low MW by-product of the grafting reaction.

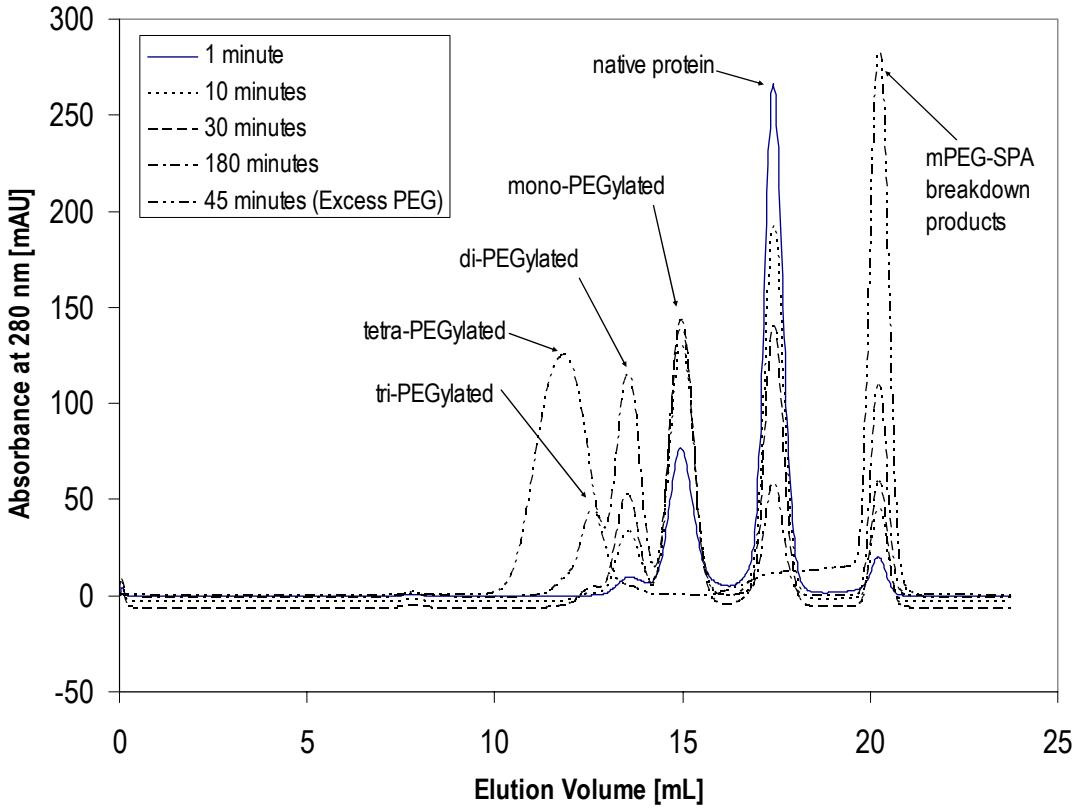

Figure 3. SEC chromatograms for samples withdrawn at intervals from batch PEGylation of α-lactalbumin with mPEG-SPA 5000, showing change in extent of PEGylation with time.

Similar chromatograms were obtained (data not shown) using a Superdex 200 column for analysing proteins PEGylated with mPEG-SPA 2000 and 20,000. Peak maxima

corresponding to the individual PEG-protein products were not resolved for mPEG-SPA 2000 except for α-lactalbumin. Similar chromatograms were also obtained (data not shown) using a Superdex 75 column for modification of α-lactalbumin with mPEG-SPA 2000 and 5000 and for modification of β-lactoglobulin with mPEG-SPA 2000.

Where peak maxima were clearly resolved, the K_{av} values of each PEG-protein reaction product identified were calculated using equation (1). In Figure 1 values for mPEG-SPA 5000 grafted proteins separated on the Superdex 200 column are plotted against their corresponding molecular weights (calculated from the native protein plus mPEG-SPA molecular weights and confirmed by MALDI-TOF). Comparison of experimental data points with the protein and PEG calibration curves in Figure 1 confirms that PEGylated proteins behave neither as proteins nor PEG molecules (of similar MW) in size exclusion media. Rather, as more PEG is grafted to a protein the related Kav shifts from the native protein's position on the protein standard curve towards the PEG standard curve (see also Fee (*13*)).

Clearly the distribution coefficients of the PEG-proteins in Figure 1 depend on the molecular weights of both PEG reagents and native proteins, as well as the PEGylation extent. However the effects of increasing *N* (and the total mass of PEG grafted) appears to correlate for samples related to each type of native protein. This suggests existence of a general method to relate such distribution coefficients to values for native protein MW, PEG reagent MW, and *N*.

It is common practice to calibrate SEC columns using molecular weight (M_r) standards and to produce a calibration chart in terms of the K_{av} versus $\ln(M_r)$. Normally such a curve is approximately linear over a range of molecular weights for a given SEC media. However, the distribution coefficient in SEC is not governed by molecular weight but, more generally, by the hydrodynamic volume or viscosity radius, R_h, of the molecule (*18*). A universal calibration method, though not in common use for proteins, can be produced for a given SEC

media for globular proteins and flexible polymers such as PEG in terms of K_{av} versus viscosity radius (*19*).

For globular proteins, the viscosity radius (in Ångströms) of the molecule (assuming a spherical shape) is related to its molecular weight in Da, by equation (2) (*20*).

$$R_{h,prot} \approx (0.82 \pm 0.02) M_r^{1/3} \qquad (2)$$

Kuga (*21*) collated data from fourteen authors on the limiting viscosity numbers and diffusion coefficients of a variety of polymers in water, including PEG, and used these to calculate the R_h of the equivalent sphere for each compound. The radii calculated from viscosity and diffusion data agreed well with each other when correlated to MW across six orders of magnitude. A subset of Kuga's data for PEG's of MW 0.2 to 1200 kDa yields the following power-law relationship (correlation coefficient $R^2 = 0.9995$) between viscosity radius (Å) and molecular weight (Da) (Figure 4):

$$R_{h,PEG} = 0.1912 M_r^{0.559}. \qquad (3)$$

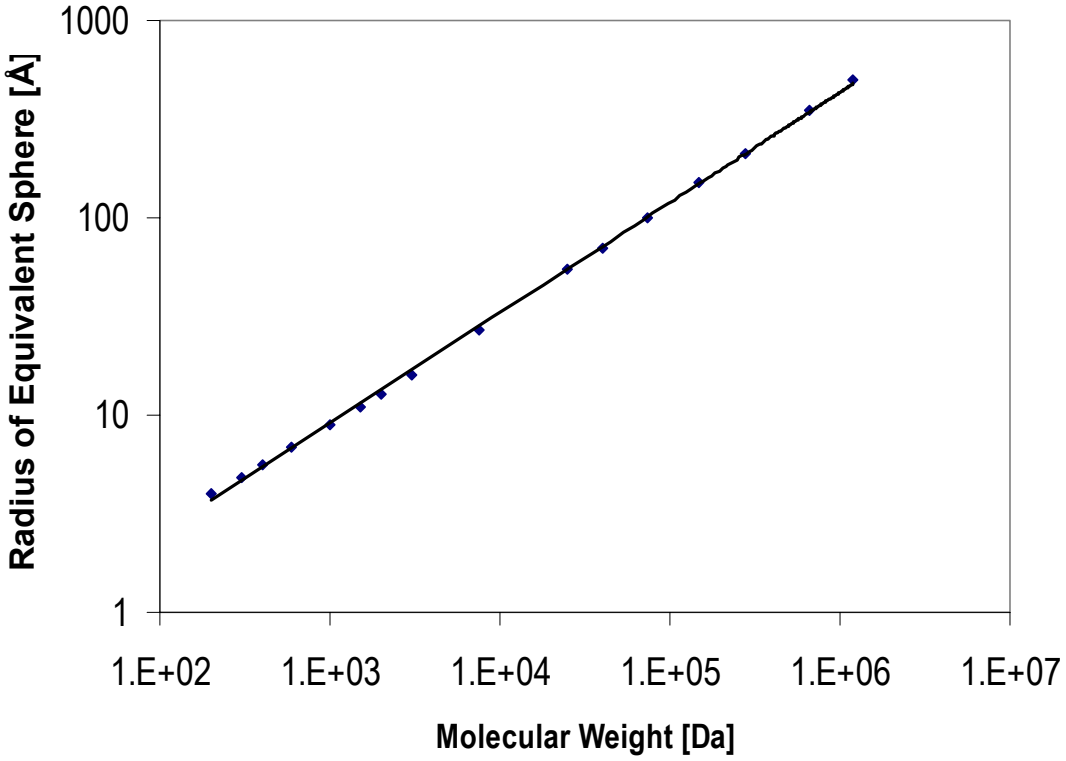

Figure 4. Viscosity radius of PEG molecules versus molecular weight (after Kuga et al. (*21*)).

The viscosity radii of all proteins and PEG's studied were calculated using equations (2) and (3), respectively, and a universal calibration curve was produced for each SEC column studied. Figure 5 shows the "universal" calibration curve for Superdex 200 HR 10/30. A similar curve was obtained for the Superdex 75 column (data not shown).

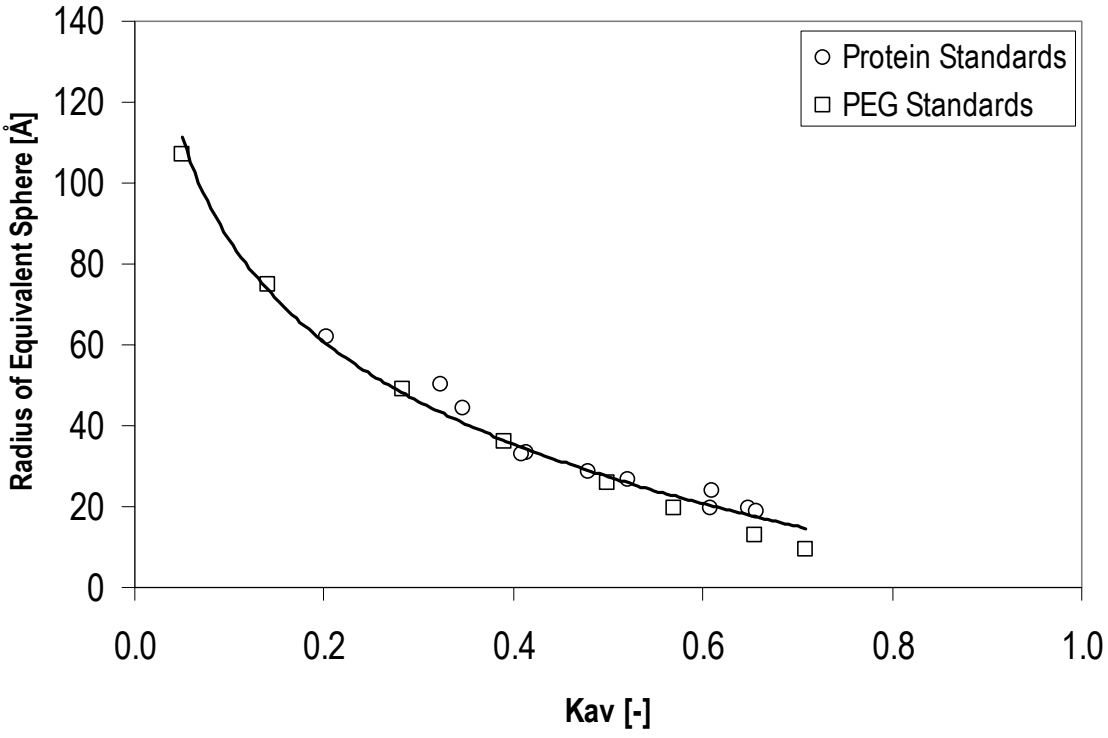

Figure 5. Universal calibration curve, viscosity radius versus K_{av} for PEG and protein molecular weight standards.

Figure 5 shows that, in keeping with Kunitani et al. (*19*), the PEG in water standard data noted above falls on the same universal curve as for protein standards in PBS. Although the relative size of PEG polymer random coils is expected to be affected by aqueous solution temperature, salt concentration and salt type, one might not expect much difference in hydration radius and SEC behaviour between PEG's in PBS and distilled water at room temperature, since both conditions lie far from lower critical solution conditions. Under similar conditions, when characterising ion exchange media, Hunter and Carta (*22*) showed that ionic strength had no effect on the retention volume of neutral flexible polymers. We therefore conclude that the sizes of PEG's in phosphate buffered saline are approximately the same as they are in water. Since the viscosity radii of both proteins and PEG molecules lie on the same universal calibration curve for a given SEC media it is reasonable to expect that

PEGylated proteins will also conform to the same curve. It should therefore be possible to experimentally estimate the viscosity radii of PEG-proteins from their elution volumes in an SEC column calibrated in this way.

Table 1. Properties of proteins PEGylated with mPEG-SPA 2000.

Protein	*N*	MW expected	MW by MALDI -tof[b]	R_h Predicted (Å) Eqtn (5)	R_h Predicted (Å) Eqtn (11)	R_h Expt Superdex 200 (Å)	R_h Expt Superdex 75 (Å)	Volume of PEG layer ($Å^3$)	Surface Area to Volume Ratio of PEG layer ($Å^{-1}$)
α–lactalbumin	0	14200	14200	-	19.8	20.1	18.4	0	-
	1	16500	16500	27.0	26.2	25.1	23.7	34259	0.232
	2	18800	18800	29.9	30.3	28.6	28.1	76758	0.144
	3	21100	21200	32.8	34.1	31.2	31.0	126459	0.112
β–lactoglobulin	0	37400	18600[c] (37400)	-	27.3	25.7	25.4	0	-
	1	39700	20700[c] (39300)	34.2	33.3	[d]	-	45859	0.272
	2	42000	23100[c] (41700)	37.1	36.9	[d]	34.7	98576	0.158
	3	44300	25400[c] (44000)	39.9	40.1	37.3	-	157552	0.119
BSA	0	66700	66700	-	33.1	34.5	-	0	-
	1	69000	69000	38.4	38.9	[d]	-	55424	0.306
	2	71300	71100	41.1	42.2	39.6	-	116902	0.172
	4	73600	73400	46.3	48.0	44.6	-	256476	0.104
	8	85100	85200	56.9	58.2	57.6	-	594629	0.067

[b] Rounded average values from all fractions assayed.

[c] Values correspond to monomer. Values indicated in brackets correspond to the measured value plus the native protein monomer molecular weight.

[d] No peak maxima on chromatograms but MALDI-tof analysis of fractions indicated molecular weights that were consistent with those expected.

Table 2. Properties of proteins PEGylated with mPEG-SPA 5000.

Protein	*N*	MW expected	MW by MALDI-tof[b]	R_h Predicted (Å) Eqtn (5)	R_h Predicted (Å) Eqtn (11)	R_h Expt Superdex 200 (Å)	R_h Expt Superdex 75 (Å)	Volume of PEG layer ($Å^3$)	Surface Area to Volume Ratio of PEG layer ($Å^{-1}$)
α–lactalbumin	0	14200	14200	-	19.8	20.1	18.4	0	-
	1	19800	19900	30.8	31.7	31.2	30.2	99668	0.126
	2	25400	15500	37.6	40.1	39.1	38.7	242024	0.084
	3	31000	30800	44.3	47.7	46.2	-	419090	0.068
	4	36600	36700	51.0	54.6	53.0	-	626448	0.058
β–lactoglobulin	0	37400	18600[c] (37400)	-	27.3	26.2	-	0	-
	1	43000	24400[c] (43000)	38.4	38.1	38.2	-	126019	0.136
	2	48600	29900[c] (48500)	45.1	45.3	44.9	-	289379	0.087
	3	54200	35400[c] (54000)	51.8	51.9	51.5	-	484617	0.069
BSA	0	66700	66700	-	33.1	33.7	-	0	-
	1	72300	72500	41.9	43.3	41.7	-	148310	0.146
	2	77900	77900	48.1	49.9	48.7	-	330470	0.090
	3	83500	83500	54.3	55.9	55.0	-	542378	0.070

Tables 1 to 3 show viscosity radii data for all of the PEG's, proteins and PEG-proteins studied in this work, as determined from the above universal calibration curves using K_{av} values calculated from experimentally determined elution volumes.

Two methods and related models for predicting the viscosity radii of PEG-proteins will now be described. We must be cautious in interpreting the viscosity radius too literally in terms of an actual physical radius. Chromatography is a stochastic process, so the hydrodynamic radius that is calculated from chromatography data represents an average of many molecular level interactions. The viscosity radius throughout this paper refers to the radius of an equivalent sphere in aqueous solution, whatever the true shape of the molecule. However, as a starting point for analysis we consider the radius, $R_{h,PEGprot}$, of a molecule formed by the conjugation of a protein of radius $R_{h,protein}$ and a PEG molecule of radius $R_{h,PEG}$.

Table 3. Properties of proteins PEGylated with mPEG-SPA 20,000.

Protein	*N*	MW expected	MW by MALDI-tof[b]	R_h Predicted (Å) Eqtn (5)	R_h Predicted (Å) Eqtn (11)	R_h Expt Superdex 200 (Å)	Volume of PEG layer (Å^3)	Surface Area to Volume Ratio of PEG layer (Å^{-1})
α–lactalbumin	0	14200	14200	-	19.8	19.6	0	-
	1	36600	36500	51.1	54.4	52.3	704985	0.056
	2	59000	58600	78.1	77.6	77.0	1934851	0.039
β–lactoglobulin	0	37400	18600[c] (37400)	-	27.3	26.3	0	-
	1	59800	40700[c] (59300)	58.6	57.9	66.6 58.8[e]	792176	0.056
	2	82200	62600[c] (81200)	85.6	79.6	82.4 77.4[+++]	2079600	0.039
BSA	0	66700	66700	-	33.1	33.3	0	-
	1	89100	88600	60.5	61.5	61.7	1022470	0.048
	2	111500	110400	85.3	81.8	84.1	2364463	0.036

Model 1

We assume that the generally benign effect of PEG grafting on protein activity (and the fact that PEG is a common food additive and is one of the compounds listed by the US Food and Drug Administration as "generally regarded as safe") indicates that the general shape (viscosity radius) of a protein is maintained in the PEG-protein conjugate. Now observe that in the aqueous environments related to this study the experimentally determined viscosity radius (R_h = 53.0 Å) of α-lactalbumin modified with four mPEG-SPA 5000 molecules (Table 2) is similar to that of α-lactalbumin modified with one mPEG-SPA 20,000 (R_h = 52.3 Å, Table 3). This implies that the final molecular size for each PEG-protein species is determined by native globular protein size and the total amount of PEG grafted, rather than

[e] Values obtained after fractionating the mono-PEGylated peak and re-running individually.

by PEG reagent molecular weight or degree of grafting. We have observed the same behaviour for cytochrome C modified with mPEG-NHS reagents of 10,000, 20,000 and 40,000 kDa, wherein PEGylated species with the same total amount of PEG grafted per protein molecule elute during SEC at the same volume, regardless of *N* (data not shown).

It follows from the previous paragraph that the excess (or difference in) viscosity radii between a native protein and PEGylated forms of that protein can be expressed as a function of the total PEG molecular weight, $M_{r,totPEG}$. The simplest function is a linear one such that

$$R_{h,PEGprot} - R_{h,protein} = a + bM_{r,totPEG} \quad (4)$$

where *a* and *b* are constants. Figure 6 shows that a plot of ($R_{h,PEGprot} - R_{h,protein}$) vs $M_{r,totPEG}$ yields the expected linear relationship for each protein, with a regression coefficient $R^2 > 0.99$ in each case. Therefore the viscosity radius (Å) of a PEGylated protein is given by

$$R_{h,PEGprot} = 0.82\left(M_{r,prot}\right)^{0.33} + a + bM_{r,PEGtot} \quad (5)$$

where *a* = 4.88, 5.21 and 3.73 Å for α-lactalbumin, β-lactoglobulin and BSA respectively, and *b* = 0.0012 Å/Da for α-lactalbumin and β-lactoglobulin and *b* = 0.0011 Å/Da for BSA.

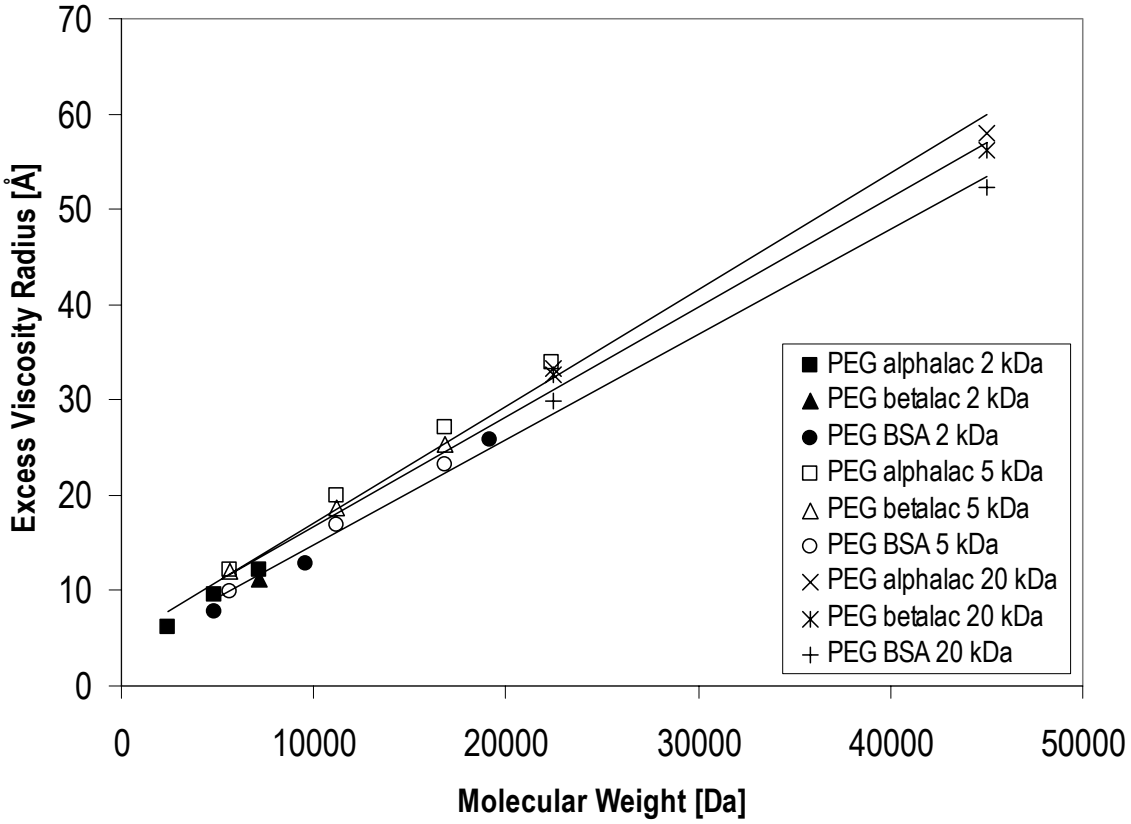

Figure 6. Excess radii of PEGylated proteins versus the total molecular weight of conjugated PEG.

Intuitively, we expect the value of *a* to be 0 i.e. as the molecular weight of total grafted PEG approaches zero, so should the excess radius. It may be that the relationship between excess radius and total PEG molecular weight is actually weakly non-linear. The above values of *a* correspond to a discrepancy of approximately 100 to 300 Da. If the relationship is indeed linear, this non-zero intercept might be due to several factors including (a) the length of the amide linkage (MW 43) formed between the PEG and the protein, (b) covalent grafting altering the contribution (via freedom of movement and hydration) of a few ethylene oxide units (-CH2-CH2-O-, MW 44) to the overall structure, (c) the grafting reaction and localisation of PEG at the protein surface slightly altering the protein conformation and thus

its contribution to the viscosity radius of the PEG-protein, (d) weak interactions between a few polymer units of the PEG and the protein surface.

Tables 1 to 3 show that the experimentally determined viscosity radii and those predicted by equation (5) are in excellent agreement (< 3% absolute average error) in relation to both the Superdex 75 and 200 SEC column measurements.

In Tables 1 to 3, experimental values are given only for those cases where individual peak maxima were resolved. In the case of grafting with mPEG-SPA 2000, neither of the two SEC columns was able to resolve individual peaks for PEGylated β-lactoglobulin and BSA that differed by less than $N = 2$. This is not unexpected, as the K_{av} values for species differing by only a 2 kDa PEG are not large enough to obtain peak resolution in these media. However, MALDI-TOF analysis of fractions collected during elution confirmed that the elution volumes of the PEG-protein species corresponded to those expected of molecules with viscosity radii calculated from equation (5) (Table 1).

In all cases, except mPEG-SPA 20,000-grafted β-lactoglobulin, SEC elution volumes corresponded to those expected from molecular species having viscosity radii calculated from equation (5). This occurred even when chromatographed samples contained native protein, several PEG-protein species, unreacted mPEG-SPA reagent, and reaction by-products. This suggests little interaction between the species when on the column.

In the case of mPEG 20,000-β-lactoglobulin (Table 3) the initial elution volume of the mono-PEGylated protein ($N = 1$) corresponded to a viscosity radius of 66.6 Å, versus 58.6Å predicted from equation (5). The elution volume for $N = 2$ corresponded to a viscosity radius of 82.4 Å, versus 85.6 Å predicted. Both peaks showed significant tailing. Such results were very reproducible and, when grafting reactions were repeated, the products yielded the same result. MALDI-TOF analysis confirmed the identities of the products in these peaks to be mono- and di-PEGylated β-lactoglobulin dimers (Table 3).

β-lactoglobulin consists of two 18.7 kDa monomeric chains held together by Ca^{+2} mediated linkages. It does not appear that PEGylation resulted in multiply PEGylated protein monomers, as SEC determined viscosity radii did not correspond to any possible combinations of PEGylated monomeric β-lactoglobulin proteins.

A new hypothesis suggested that different PEGylated β-lactoglobulin proteins associated weakly with one another in the early regions of the SEC, chromatographing initially as a larger aggregate and then separately during their migration down the column. Fresh PEG-protein samples were run again and the peaks collected separately before being subjected to individual SEC runs. The viscosity radius of the $N = 1$ species (58.8 Å) calculated from the elution volumes agreed well with the 58.6 Å prediction from equation (5). The viscosity radius of the N = 2 species (77.4 Å from SEC) was smaller than the equation (5) related value of 85.6 Å. Such discrepancies were only seen with one reagent and one dimeric protein. They are noted here to illustrate that the relations described here may not apply to certain proteins under some circumstances.

Model 2

Although equation (5) appears to accurately predict viscosity radii and the values of the constants *a* and *b* are closely similar between protein species, optimal accuracy demands that unique values of *a* and *b* should be determined experimentally for each protein. In developing model 2 we sought a predictive method that does not depend on experimentally determined parameters. We again picture a scenario in which the protein molecule remains unchanged in size and the final size of the conjugate is determined by alterations to the conformation of the PEG molecule alone. Calculating the volume of the equivalent sphere from the viscosity radius and subtracting the volume of the equivalent sphere for the native protein gives the volume that can be attributed to the PEG portion of the conjugated molecule.

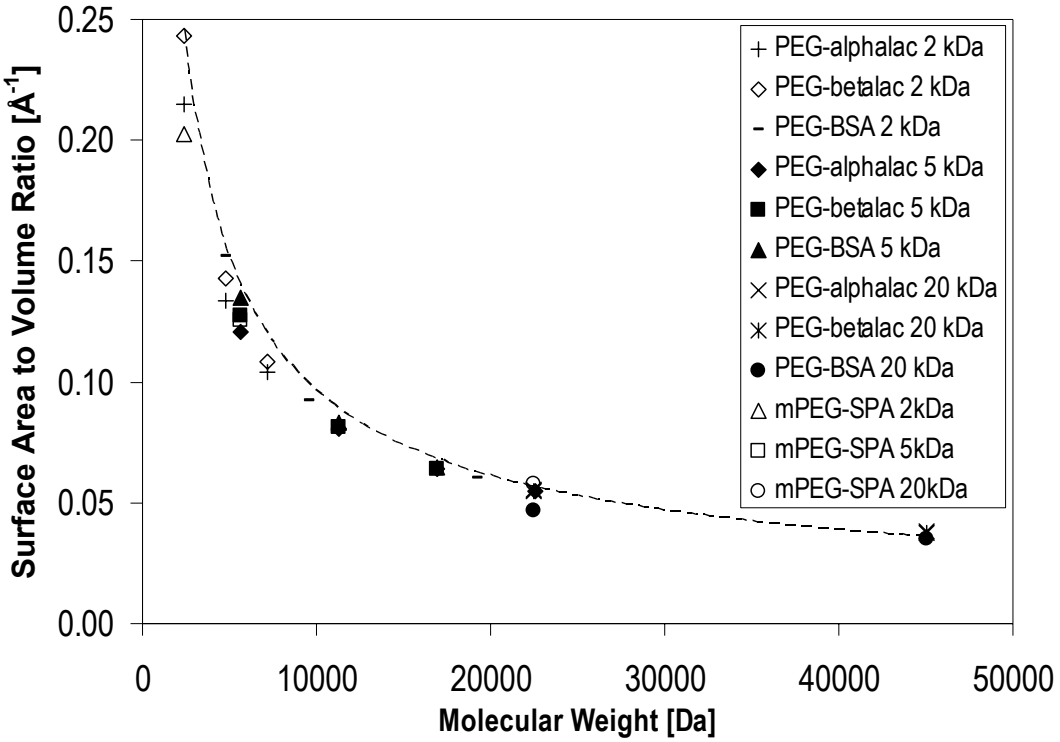

Figure 7. Surface area to volume ratio for the PEG molecules and for the PEG layer surrounding PEGylated proteins (line from equation (6)).

The volumes of the equivalent spheres for mPEG-SPA 2000, 5000 and 20,000 in PBS are 13,667 $Å^3$, 57263 $Å^3$ and 582,896 $Å^3$, respectively. Tables 1 to 3 show that the volume added to the protein molecule by the first PEG is much larger than the volume of the original PEG molecule in free solution. Also, each successive PEG group added to the conjugate molecule contributes an increasingly larger volume to the conjugate. However, if we examine the outer surface area of the conjugate and the volume contributed by the PEG, we find that the ratio of the surface area of the PEG exposed to the ionic environment to the PEG layer volume remains virtually constant for a given total PEG molecular weight (Tables 1 to 3). Figure 7 shows the surface area to volume ratio for a given total molecular weight of PEG is approximately constant both for the individual PEG reagents in free solution and for the PEG

layers surrounding all three protein species PEGylated to any extent, although the ratio increases slightly with increasing molecular weight of the native protein.

These observations, together with the fact that the final radius is determined by the total molecular weight of conjugated PEG and not by the number of PEG molecules conjugated, strongly suggests that the PEG forms a continuous layer surrounding the protein and that it adjusts its thickness such that the surface area to volume ratio of the layer is approximately the same as that of a PEG molecule with the equivalent molecular weight in free solution. Rather than a rigid coating, we envisage a very dynamic and flexible PEG molecule with enough surface interaction to result in what is in effect a protein with significant surface masking by PEG rather than a PEG spheroid grafted to a protein spheroid. Such a conformation would allow protein surface diffusion and the approach of other molecules, though such movement would be subject to some steric hindrance. The PEG layer mobility would presumably be most constrained near the site of conjugation. The protein would therefore maintain its biological function but with its *in vitro* activity reduced by an amount dependent on the position(s) of conjugation relative to the active site(s) of the protein. This view is consistent with the widely reported effects of the PEGylation location on protein activity. This is discussed further below.

We can now use the fact that under the low conductivity, room temperature conditions studied, the surface area to volume ratio is approximately constant for any given total molecular weight of PEG, to calculate the thickness of the PEG layer surrounding a PEGylated protein and thus its final viscosity radius. The ratio, AV_{PEG}, of surface area to volume of the equivalent sphere for a PEG molecule in free solution is

$$AV_{PEG} = \frac{3}{R_{h,PEG}} \tag{6}$$

and the surface area, $A_{PEGprot}$, of the PEGylated protein exposed to the phosphate buffered saline environment is

$$A_{PEGprot} = 4\pi\left(R_{h,PEGprot}\right)^2 \tag{7}$$

where $R_{h,PEGprot}$ is the viscosity radius of the PEGylated protein. The volume of the PEG fraction of the PEGylated protein, assuming that the protein volume is unchanged by PEGylation, is

$$V_{PEGlayer} = \frac{4\pi}{3}\left({R_{h,PEGprot}}^3 - {R_{h,prot}}^3 \right). \tag{8}$$

The surface area to volume ratio of the PEG layer in the PEGylated protein, $AV_{PEGprot}$, is obtained by combining equations (6), (7) and (8), noting that $R_{h,PEG}$ is the viscosity radius of the equivalent sphere for a PEG molecule having a molecular weight equal to the total molecular weight of PEG conjugated to the protein, yielding

$$AV_{PEGlayer} = \frac{{R_{h,PEGprot}}^2}{\left({R_{h,PEGprot}}^3 - {R_{h,prot}}^3\right)} = \frac{1}{R_{h,PEG}}. \tag{9}$$

Equation (9) can be rearranged to give a cubic equation in $R_{h,PEGprot}$

$$R_{h,PEGprot}{}^3 - R_{h,PEG} R_{h,PEGprot}{}^2 - R_{h,prot}{}^3 = 0 \,. \qquad (10)$$

Equation (10) has only one real root, given by equation (11).

$$R_{h,PEGprot} = \frac{1}{6}\left[108R_{h,prot}{}^3 + 8R_{h,PEG}{}^3 + 12\left(81R_{h,prot}{}^6 + 12R_{h,prot}{}^3 R_{h,PEG}{}^3\right)^{1/2}\right]^{1/3} + \frac{2}{3}\frac{R_{h,PEG}{}^2}{\left[108R_{h,prot}{}^3 + 8R_{h,PEG}{}^3 + 12\left(81R_{h,prot}{}^6 + 12R_{h,prot}{}^3 R_{h,PEG}{}^3\right)^{1/2}\right]^{1/3}} + \frac{1}{3}R_{h,PEG} \qquad (11)$$

Equation (11) shows that the viscosity radius of the PEGylated protein is a function only of the viscosity radius of the native protein and that of an equivalent sphere for a PEG molecule having a molecular weight equal to the total PEG molecular weight conjugated to the protein. Using equations (2) and (3) to substitute for $R_{h,prot}$ and $R_{h,PEG}$ in terms of molecular weight allows equation (11) to be solved for any total amount of PEG added to a given protein. Figure 8 shows the correlation between predictions using equation (11) and experimental measurements for each of the PEGylated species studied. The fit is very good (marginally better than when using equation (5), with average absolute errors of less than 3%). So too, the values predicted by equation (11) agree well with the experimentally determined values for both mono- and di-PEGylated β-lactoglobulin (with mPEG-SPA 20,000) when these species were analysed separately (Table 3).

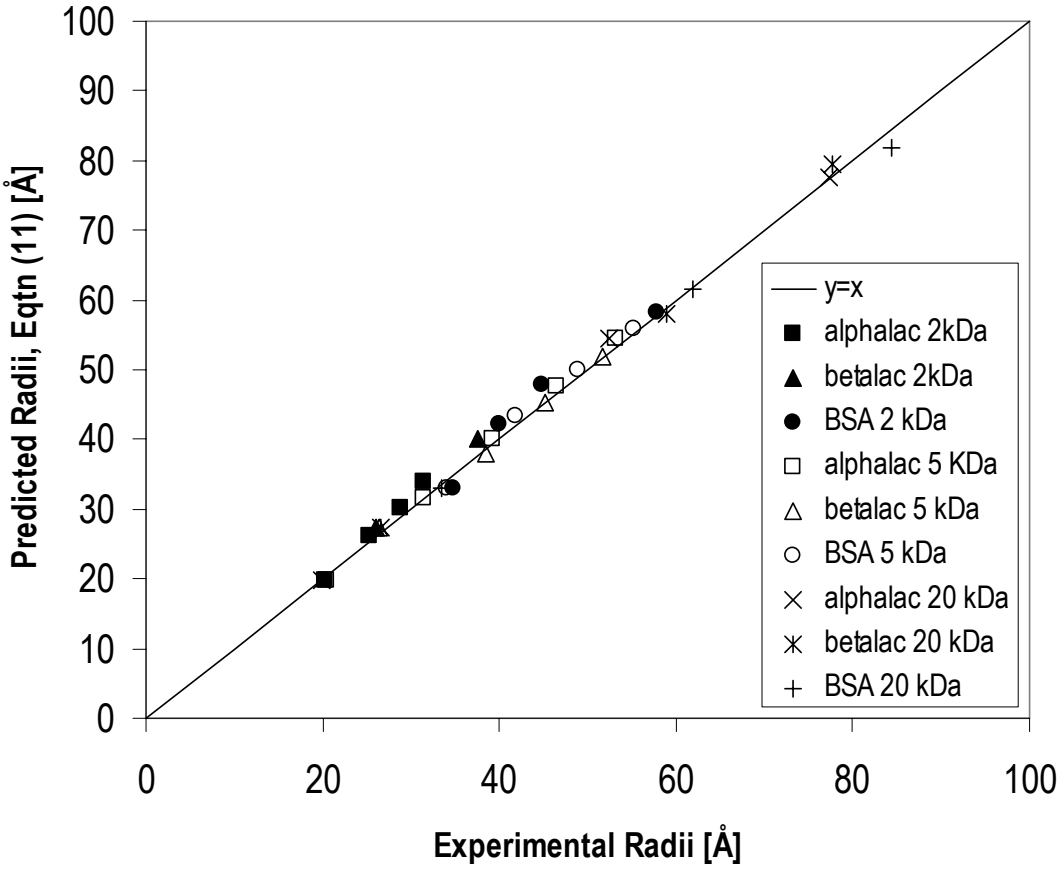

Figure 8. Comparison between experimentally determined viscosity radii of PEGylated proteins and those predicted by equation (11).

Either equation (5) or equation (11) can be used to calculate $R_{h,PEGprot}$. Equation (5) is simpler to calculate but requires experimental determination of the constants *a* and *b*. It is beyond the scope of this paper to determine the dependence of *a* and *b* on the protein species but we surmise, based upon our experience with the three proteins used in this study, that these constants are not strongly affected by protein type. Equation (11) possibly represents a more robust model of the physical situation and requires no experimentally determined parameters.

Finally, the K_{av} (and therefore the elution volume) of any PEGylated species may be predicted from either equation (5) or equation (11) together with the universal calibration curve for a given SEC column.

The general shape and size of PEG random coils appear to be dictated by polymer hydration and excluded volume (HEV) effects (*23, 24*) and to a somewhat lesser degree by interaction with solution ions (*25*). Thus above a few thousand MW, PEG's take on random coil spherical shapes that, due to weak segment-segment interactions, are more flexible and relatively larger than for proteins of similar MW. It is now well appreciated that PEG segments are capable of assuming conformations with considerable variation in relative hydrophilicity and hydrophobicity (*23, 26, 27*). When PEG molecules are attached to a surface they lose conformational freedom and in the case of a well-hydrating environment and a surface which does not exhibit strong interactions with the polymer, this is expected to lead to entropic repulsion, such that the PEG chains extend normal to the surface (*23, 28*). Such surface structures are influenced by polymer MW and surface grafting density (*28, 29*). Terminally grafted PEG conformations may vary from essentially spherical to extended "brush" conformations depending on grafting density. In the case of PEG's of 5000 to 6000 MW this may involve polymer layer thicknesses of 40 to 100 Å (*29*). Naturally there are conditions (raised salt concentration, temperature and pressure) where the polymer can be induced to interact more favourably with the surface and affect its surface conformation as well as protein adsorption.

Under the physiological salt, room temperature and atmospheric pressure conditions of the present study, PEG interactions with proteins might be expected to be dominated by HEV effects and therefore to be repulsive (*23, 29-33*). Such behaviour is believed to be responsible for the ability of PEG-coated surfaces to reduce protein adsorption, and for PEGylated proteins to show enhanced biocompatibility, serum circulation lifetime and

resistance to enzymatic degradation. Thus one might expect low-density single-point conjugation of PEG to a protein to yield a complex product composed of two linked but distinct molecular spheroids.

In contrast, the present study strongly suggests that the protein and conjugated PEG favourably interact to create a single spheroid PEG-protein complex, with the PEG portion forming a layer surrounding the protein. This is supported in particular by the excellent predictions of viscosity radius obtained by equation (11). Also, the viscosity radius of a protein conjugated with distinct PEG spheroids would tend to be increasingly independent of N above a value of 2 or 3, since each additional PEG group added would likely be contained within the swept volume of a rotating protein molecule with a PEG spheroid grafted on opposite sides. Tables 1 to 3 and figure 6 clearly show that this is not the case but that the excess radii of PEGylated proteins are linearly related to N for all proteins studied, including up to the case of $N = 8$ for BSA PEGylated with mPEG 2000. The shape and other properties of PEG in the outer layer appear to be influenced by the same HEV factors experienced by free PEG in solution, as the surface area to volume ratio of the PEG remains approximately constant between the free solution and conjugated forms. However, the volume of the layer is much larger than that for the free PEG in solution, indicating increased overall hydration (though not necessarily uniform throughout the layer).

Partition of proteins such as BSA and lactalbumin into aqueous phases rich in PEG is well documented (*31, 34, 35*) particularly in the presence other polymers such as dextran. We considered the possibility that the Superdex media, being dextran based, might provide a unique partitioning environment that caused the PEG layer to preferentially associate with the protein rather than the matrix. However, we obtained identical results (not shown) when using a Superose 12 SEC media, which is agarose based. While this does not preclude the

possibility of such partitioning, it adds confidence to the general relevance of the results obtained in the Superdex media.

It may be wrong to relate the interactions of PEG and protein molecules when each is free in solution to their interactions when conjugated, as the latter case involves a closest point of contact within their mutual hydration shells and double layers. Instead, it is possible to speculate on a variety of favourable PEG to protein interactions such as hydrogen bonding, divalent cation chelation, hydrophobic interactions, and so on (*29, 31, 33*).

It is beyond the scope of this paper to model such behaviour but it would be of great interest to know if such a model were theoretically possible and, if so, what would be its implications for the biomedical properties of PEGylated proteins. The concept of PEGylated proteins consisting of conjugated but distinct spheroids is not in keeping with biomedical results (*32, 36, 37*) which suggest that PEGylation even with relatively small MW PEG's can dramatically alter a protein's average surface properties. Such results are more in keeping with the single spheroid concept assumed in the present study in deriving equation (11).

Conclusions

When molecular weight is used to calibrate a SEC column, PEGylated proteins apparently behave differently from both pure proteins and pure PEG molecules in size exclusion media. However, the distribution coefficients of protein and PEG macromolecules in size exclusion chromatography columns are related to their hydrodynamic volumes or viscosity radii such that a single universal calibration curve can be created for each column. The relationship between the distribution coefficient and viscosity radius apparently extends also to

PEGylated proteins and thus the viscosity radii of these proteins can be determined experimentally from a column calibrated with PEG and/or protein standards alone.

The viscosity radius of PEGylated proteins in phosphate buffered saline is dependent on the native protein size and the total amount of PEG conjugated but is independent of the PEGylation extent, *N*. There is a linear relationship between the effective radius added to a protein and the total molecular weight of grafted PEG.

Based on the assumption that the native protein conformation is unaffected by PEGylation, it is proposed that the conjugated PEG acts to form a dynamic, hydrated polymer-rich layer at the protein surface whose thickness is such that it maintains an external surface area to volume ratio (i.e. the surface area exposed to the external ionic environment divided by the volume of the PEG layer) approximately equal to that of a PEG molecule of equivalent molecular weight in free solution.

Two equations for predicting the viscosity radii of PEGylated proteins in PBS, an ionic environment that closely matches physiological conditions, have been presented. Equation (5), based on the simple assumption that PEG-protein radius is linearly related to the total conjugated PEG molecular weight predicts viscosity radii to within 3% of experimental values. Equation (11) is based on the assumption of a constant protein molecular conformation, together with a constant PEG layer surface area to volume ratio. The latter method is more representative of the physical situation and predicts the viscosity radii well, without the need for experimentally determined parameters.

The K_{av} (and therefore the elution volume) of any PEG-protein species may be predicted from either equation (5) or equation (11) together with the universal calibration curve for a given SEC column.

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