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Analytical partitioning of poly(ethylene glycol)-modified proteins

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Abstract

Covalently grafting proteins with varying numbers (n) of poly(ethylene glycol) molecules (PEGs) often enhances their biomedical and industrial usefulness. Partition between the phases in aqueous polymer two-phase systems can be used to rapidly characterize polymer–protein conjugates in a manner related to various enhancements. The logarithm of the partition coefficient (K) approximates linearity over the range $0<n<x$. However, x varies with the nature of the conjugate (e.g., protein molecular mass) and such data analysis does not facilitate the comparison of varied conjugates. The known behavior of surface localized PEGs suggests a better correlation should exist between log K and the weight fraction of polymer in PEG–protein conjugates. Data from four independent studies involving three proteins (granulocyte–macrophage colony stimulation factor, bovine serum albumin and immunoglobulin G) has been found to support this hypothesis. Although somewhat simplistic, 'weight fraction' based analysis of partition data appears robust enough to accommodate laboratory to laboratory variation in protein, polymer and phase system type. It also facilitates comparisons between partition data involving disparate polymer–protein conjugates.

Keywords: Partitioning; Aqueous two-phase systems; Poly(ethylene glycol); Proteins; Immunoglobulin; Bovine serum albumin

1. Introduction

Covalent modification of therapeutic proteins, pharmaceutical molecules and drug carrying colloids, with neutral polymers such as poly(ethylene glycol) (PEG) is a method of choice to overcome many problems associated with their use in vivo. PEG–protein conjugates typically exhibit extended plasma half-life, reduced immunogenicity, increased solubility, and enhanced resistance to proteolysis [1–4]. PEG-modified liposomes and other drug carriers display similarly enhanced properties [2,5–7]. PEG-modification also enhances thermostability, solvent dependency plus other properties related to industrial applications [1–3,8,9]. PEG is ideal for tethering proteins and other molecules to each other, or to surfaces such as chromatographic supports and biosensors [2]. While a number of standard chemical methods are available to separate and analyze PEG-conjugates [1–9] there is a recognized need to develop new methods. Such methods should be non-destructive, rapid, cost-effective and able to differentiate PEG-modified substances in a manner which relates to their enhanced capabilities [1,2]. Capa-

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bilities which often relate to PEG molecules 'masking' immunogenic groups [9] or otherwise altering the surface properties of PEG-modified substances [1–8].

Mixing neutral polymers such as PEG and dextran polyglucose in aqueous buffered solution at low concentrations (e.g., 4% w/w) typically results in the formation of an aqueous polymer two-phase system consisting of a PEG-rich phase floating on top of a denser dextran-rich phase. Delicate particles, proteins and molecules readily partition between such phases in a non-denaturing manner. The partition coefficient (K) of material between the phases reflects various features of the substance–solution interface (e.g., net interfacial free energy in the two phases) and many studies have correlated log K and various physical or physiological attributes of proteins, cells and other substances [10–13]. In addition log K has also been shown to vary directly with polymer modification of proteins or other substances via covalent [1,2,10,11,14–18] or other means, e.g., affinity interactions [2,10–13,19,20]. Partition rapidly provides semi-quantitative information involving both the degree of polymer modification and its ability to alter protein surface features. Information whose investigation by other means may be more costly, slower, technically demanding and possibly involve animal testing. Partition is expected to be indicative of the effectiveness of polymers of differing type, branching, molecular weight and functionality, when used at different degrees of grafting, to protect and 'mask' modified substances in a range of aqueous environments. Partition is also an ideal method for large scale separations [2,10–12]. It is therefore important to understand the relationship between K and PEG modification of macromolecules.

Earlier attempts to address the relationship between K and the number of PEG molecules (n) localized at a protein via covalent or affinity methods [14–21] focused on a linear relationship. However, the linear relationship, predicted on the assumption that each grafted PEG contributed equally to K [14,20,22], did not hold over the larger ranges of n possible with covalent grafting [15,16,20]. The predictive and comparative value of log K versus n analysis is currently limited to interpolation [18,21,23]. This reduces the use of such easily obtained partition data to predict and compare the properties and behavior of polymer-modified proteins.

In the present study log K versus n data from four independent studies involving three types of PEG-modified proteins, was evaluated in order to identify a simple laboratory correlate which is free of the above limitations. The proteins were granulocyte–macrophage colony stimulation factor (GM-CSF), bovine serum albumin (BSA) and immunoglobulin G (IgG). The partition data was published in four papers in different journals and data formats, by three independent groups [15,16,18,23]. In addition to varied protein types, the studies involved different molecular weights of PEG, sources of PEG, PEG coupling chemistry, phase systems and methods of determining both n and K. In spite of such differences current knowledge of the behavior of interfacially localized PEG allowed identification of a direct relationship, between log K and polymer weight fraction in the PEG–protein conjugates. Use of this approach enhanced both comparative evaluation of the data, and understanding of how PEG modification may affect the surface properties of proteins in solution.

2. Experimental

The data was from experiments conducted at room temperature in accordance with standard practices [10–12] using NaCl-enriched two-phase systems containing dextran T 500 and PEG 8000 or 6000. It is summarized in Tables 1 and 2. The protein partition coefficient (K) was taken as the concentration ratio of protein in the PEG-rich phase and dextran-rich phase. Table 2 data represents mean values of three or more determinations (with standard deviations shown in some cases). Tables 1 and 2 also indicate data related to the polymer weight fraction (PWF) in the resulting conjugate. This is expressed as $[nM_1 \cdot (nM_1 + M_p)^{-1}]$ where M_1 and M_p refer to the mean molecular mass of the PEG monomer and protein, respectively [14,15,20,22]. In these calculations M_p was taken to be 14.4 kDa for GM-CSF [23,26], 66.2 kDa for BSA [4,27] and 160.0 for kDa IgG [28]. For ease of comparison original data was converted to log K versus n format (Table 2). The IgG and BSA partition data of Karr et

al. [16] was converted from % of amino groups substituted to n values based on 60 [4,27] and 90 [29] amino groups per BSA and IgG molecule, respectively. Table 2 data were plotted and subjected to least squares fit analysis (Table 3) using Cricket Graph version 1.3.2, (Cricket Graph Software, Malvern, PA, USA) on a Macintosh computer. Some details of individual protein modification and partition studies are given below.

Karr et al. [16] prepared PEG–BSA or PEG–IgG conjugates by reacting various amounts of cyanuric chloride [4] activated monomethoxy-PEG 5000 with 4 mg ml^{-1} of BSA (Sigma) or IgG (sheep anti-human red blood cell) in 100 mM sodium borate buffer (pH 9.2), under stirring for 1 h. Unattached PEG was removed by 30 kDa exclusion diafiltration (Amicon PM-30 membrane) with 50 mM borate buffer. Free amino groups were estimated via reaction with sodium trinitrobenzene sulfonate in 4% (w/w) sodium bicarbonate (modified Habeeb method) which is dependent on removal of unreacted PEG [16,24]. Partition was determined in a phase system consisting of 5.0% (w/w) dextran T-500 (M_r=500 000 from light scattering, Pharmacia and Upjohn), 4.0% (w/w) PEG 8000 (M_r=6650 from gel permeation chromatography [13], Union Carbide), 150 mM NaCl, 7.3 mM Na_2HPO_4, 2.3 mM NaH_2PO_4 (pH 7.2). According to convention [10–13] this system was designated (5.0, 4.0) V. BSA and PEG–BSA samples (0.1 ml at 4 mg ml^{-1}) were added to 2 ml of phase system in test tubes which were then mixed twice by vortex mixing at 10-min intervals and centrifuged for 5 min at 200 g. Due to dilution the final phase system composition was expected to approximate (4.75, 3.80) V. Protein concentration in each phase was measured fluorimetrically (Turner spectrofluorometer at 280 nm excitement and 380 nm emission) with the appropriate phase as a control. IgG and PEG–IgG samples (0.2 ml at 2 mg ml^{-1}) were added to 2 ml of (5.00, 4.25) V phase system whose final composition was expected to approximate (4.55, 3.86) V. Partition was determined by UV absorption at 280 nm. Karr et al. recently reviewed various technical and other aspects of their research [24].

Sharp et al. [15] reacted cyanuric chloride activated [^{14}C]monomethoxy-PEG 1900 with ^{125}I-IgG (affinity purified, rabbit anti-human red blood cell IgG [15]) in 100 mM borate buffer (pH 9) for 40 min. PEG–IgG was separated from unreacted PEG by gel filtration. The amount of PEG attached was determined from the concentration ratio of ^{14}C to ^{125}I. Partition was determined in a (5.0, 3.4) V phase system compounded with polymers similar to those used by Karr et al. Partition experiments involved combining 2 ml volumes of each phase and ≤0.2 ml of buffer containing IgG or PEG–IgG. This resulted in a homogeneous phase to which 12 μl of 30% (w/w) PEG 8000 solution was added in order to regenerate a biphasic system whose final composition is estimated to have been (4.75, 3.41) V. After mixing and settling, top and bottom phase aliquots were analysed for protein concentration on the basis of their radioactivity.

Delgado et al. [18] reacted various amounts of tresylated [30] monomethoxy-PEG 5000 with 1.5 mg ml^{-1} BSA (Sigma) in 50 mM sodium phosphate buffer (pH 7.5), containing 125 mM NaCl for 2 h at room temperature under gentle magnetic stirring. The degree of modification (Table 2) was established by fluorescamine (4-phenylspiro[furan-2(3H), 1′-phthalan]-3,3′-dione) assay [25] and gel permeation chromatography (see below). Fluorescamine interacts with the primary amino groups of proteins (lysine residues and amino terminus) to form a fluorophore (390 nm excitation, 475 nm emission) whose fluorescence is proportional to amine concentration, and much less affected by residual PEG [19,25]. Partition involved 0.1-g protein solution being added per gram of phase system to yield a 4.75% dextran T-500 (Pharmacia and Upjohn), 4.75% PEG 6000 (British Drug Houses), 150 mM NaCl, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4 (pH 6.8) system. After 30–40 inversions, the mixture was left to settle at room temperature (15–20 min) until complete separation of the phases was observed. Top and bottom phases were analysed for protein concentration by Coomassie brilliant blue assay (Pierce).

Delgado et al. [23] reacted ^{125}I-GM-CSF (Amersham, UK) with tresylated mPEG 5000 for 2 h at room temperature. The degree of modification was established as follows. Reaction mixtures were subjected to gel permeation chromatography and partition coefficients of $(PEG)_n$–GM-CSF fractions, where n equals 1, 2 or 3, were determined in a (4.75, 4.75) V (pH 6.8) system (see above) via ^{125}I gamma

counting. The average degree of modification of whole reaction product mixtures was then determined by computer aided deconvolution of gel permeation chromatogram peaks and by fitting a protein product mixture's substitution and partition profiles (Ref. [23] and Delgado and Francis, unpublished).

3. Results and discussion

Table 1 summarizes similarities and differences in the different data analyzed [15,16,18,23]. The three proteins studied GM-CSF [14.4 kDa, 7 free (terminal and 6 γ-lysine) amine groups] [26], BSA (66.2 kDa, 60 free amine groups) [27] and IgG (160 kDa, 90 free amine groups) [28,29] represent a wide range of molecular mass and potentially reactive amine groups. They were modified with up to 2.5, 46 and 43 monomethoxy-PEG molecules to yield maximal polymer weight fractions of approximately 0.4, 0.8 and 0.6, respectively. PEG molecules were functionalized with cyanuric chloride [4] or tresylate [30] which are comparable, though somewhat different, in their specificity for and reaction with protein groups [31]. The GM-CSF, BSA and sheep IgG studies involved PEG 5000–protein conjugates in similar, albeit not identical, two-phase systems. In particular close similarity in the two BSA studies provided an opportunity to compare analogous results from different laboratories. Sharp et al. [15] partitioned PEG 1900-modified rabbit IgG in a two-phase system which was close to monophasic. Reproducibility of such results is more sensitive to polymer lot, temperature and other experimental factors [11,22]. The NaCl-enriched systems common to all four studies are expected to possess minimal electrostatic chemical potentials (e.g., 0.2 mV) [16] and to be more sensitive to polymer grafting than to slight alterations in protein surface charge (groups) [10].

Although the partition of polymer modified proteins has been of interest for almost two decades [10,14] and numerous proteins have been derivatized with PEG [1–3,31], Table 2 contains some of the most comprehensive data available on the partition of PEG–proteins as a function of polymer grafting density. Enhanced PEG-modification analysis allowed improved analysis of n values related to the work of Deglado et al. (see above); however, this data is not significantly different from that published previously [25]. Multistep countercurrent phase partition and gel chromatography often indicate heterogeneity in PEG–protein conjugates such as those related to Table 2 [1,15,18,21]. This fact, the experimental differences noted above, plus lack of information concerning the distribution and orientation of grafted PEGs in relation to protein structure limit overt analysis of the data. However, analysis of its general trends provides insight to the influence of polymer-grafting on conjugate surface properties and partition behavior.

According to theory log K should vary directly

Table 1
Summary of experimental variables

Protein	M_p[a] (kDa)	mPEG M_t	PEG group	n range	Polymer weight fraction	Phase system[b]	Ref.
GM-CSF	14.4	5000	Tresylate	0–2.5	0–0.45	(4.75, 4.75) V	[21,23]
BSA	66.2	5000	Tresylate	0–17	0–0.58	(4.75, 4.75) V	[18,23]
BSA	66.2	5000	Cyanuric chloride	0–46	0–0.79	(4.75, 3.80) V[c]	[16]
IgG	160.0	5000	Cyanuric chloride	0–42	0–0.57	(4.55, 3.86) V[c]	[16]
IgG	160.0	1900	Cyanuric chloride	0–43	0–0.31	(4.75, 3.41) V[c]	[15]

[a] See Section 2.
[b] Phase systems contained dextran T 500, PEG 8000 or 6000 and phosphate buffered saline. Polymers were different lots from different sources.
[c] Estimated by authors from information supplied in references.

Table 2
Protein partition and PEG modification data[a]

Average amino groups modified (n)	Log K partition[b]	Δ Log K partition	Polymer weight fraction
PEG 1900–IgG, from Sharp et al. [15]			
0	0.041±0.020	0.000	0.000
27	0.771±0.039	0.730	0.222
31	1.267±0.048	1.226	0.247
43	1.605±0.051	1.564	0.312
PEG 5000–IgG, from Karr et al. [16]			
0.0	0.000±0.053	0.000	0.000
42.3	0.825±0.129	0.825	0.569
PEG 5000–BSA, from Karr et al. [16]			
0.0	−0.348±0.060	0.000	0.000
6.0	0.908±0.149	1.256	0.326
23.4	1.380±0.152	1.728	0.654
28.2	1.380±0.152	1.728	0.695
45.6	1.690±N.D.	2.038	0.786
PEG 5000–BSA, from Delgado et al. [18]			
0.00	−0.376±0.040	0.000	0.000
6.16±2.77	0.446±0.063	0.822	0.332
11.29	0.787±0.098	1.163	0.477
13.59±2.71	1.066±0.098	1.442	0.523
16.76	1.070±0.057	1.446	0.575
PEG 5000–GM-CSF, from Delgado et al. [23]			
0.000	0.202±0.054	0.000	0.000
0.072	0.155±0.025	−0.047	0.024
0.643	0.338±0.007	0.136	0.179
1.211	0.391±0.036	0.189	0.292
2.062	0.459±0.045	0.257	0.412
2.526	0.608±0.015	0.406	0.462

[a] See Section 2.
[b] Mean±standard deviation.

with protein surface alteration, as reflected by protein interaction with the two aqueous environments of the system [11,22]. Figs. 1 and 2 illustrate that log K is directly related to PEG grafting ratio (n), and that appreciable shifts in partition are often associated with small variations in n. For example, Karr et al. found grafting an average of only six PEG 5000 molecules to BSA induced 90% of the protein molecules to partition into the PEG-rich phase (i.e., $\Delta\log K=1.26$) [16]. This is noteworthy as regards potential use of partition to both analyze (as well as fractionate) PEG-grafted proteins.

The sensitivity of log K to n, exhibited by PEG 5000-modified GM-CSF and BSA partitioned by

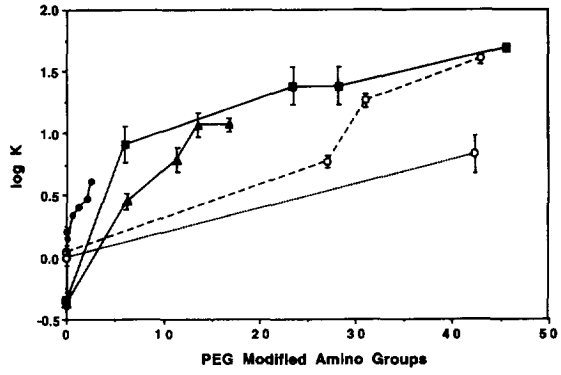

Fig. 1. Log K partition versus degree of PEG modification (Table 2) for PEG 5000–cyanuric chloride modified sheep IgG (- -○- -), PEG 5000–cyanuric chloride modified BSA (-■-), PEG 5000–tresylate modified BSA (-▲-), PEG 5000–tresylate modified GM-CSF (-●-) and PEG 1900–cyanuric chloride modified rabbit IgG (-○-).

Delgado et al. in identical phase systems, appears to vary inversely with protein size (Figs. 1 and 2). Such variation is also in keeping with the data of Karr et al. for the partition of PEG 5000-modified BSA and sheep IgG (two data points) [16].

Graphs of log K versus n graphs for the above two independent studies involving PEG 5000–BSA are quite similar over their common range of n. This suggests that partition results are similar even when experiments involve slightly different polymers, PEG-functional groups, protein samples, phase systems and laboratories in different countries. It should be noted that Delgado et al. [18] used 4.75% (w/w) of PEG 6000 while Karr et al. [16] used 3.80% of PEG 8000.

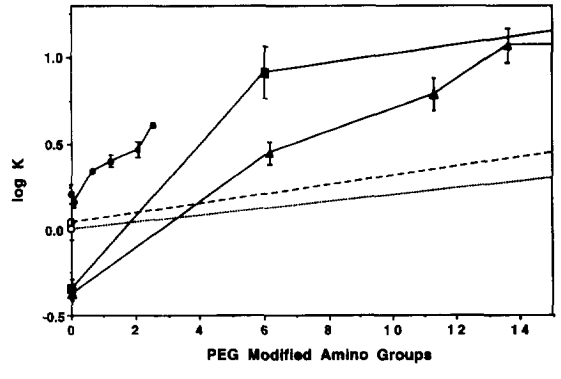

Fig. 2. Detail of Fig. 1 showing GM-CSF and BSA partition at low n values.

Although Figs. 1 and 2 suggest the relationship between log K and n approximates linearity over varying ranges of n [18,23], they question overt belief in such linearity. A linear relationship would imply equivalency of grafted PEG molecules at affecting conjugate surface features and lack of influence of grafted polymer chains on further grafting. Recent studies by many groups (discussed below) oppose such beliefs. In addition Least squares fit analysis suggests a second order equation provides a better fit (Table 3) for both sets of BSA data, which span a relatively large range of n. The best first order (linear) fit is seen for the PEG 5000–GM-CSF data which involves a relatively small range of n. This may be particularly noteworthy as regards PEG-modification of hormones, cytokines and other relatively small proteins. The next best first order fit involves the (PEG 1900–IgG) conjugate of lowest PEG-to-protein molecular weight ratio and a data set of only four points.

The conformations and interactions of PEG molecules in aqueous solution [2,32,33] or localized at various interfaces [2,34–38] are numerous, as are the non-covalent interactions which PEG molecules are capable of entering into. PEG polymer segments can interact with each other, water molecules, various ions and other chemical groups [above references]. PEG molecules may bind cations [2,33] and appear capable of hydrogen bonding with proton donors (so as to significantly increase their pK_a) [39,40]. They may exhibit hydrophobic interactions [34,37,38] based on changes in the *gauche–trans* equilibrium related to their C–O bonds [41] and attractive or repulsive interactions involving both inter- and intra-chain segments (Ref. [42], above refs.). Non-covalent interactions of 'surface' localized PEG molecules with the underlying surface are largely determined by surface chemical groups—which for a protein are varied. It seems likely that PEG molecules covalently linked to proteins may interact non-covalently with other groups on the protein. As their surface density increases PEG molecules may also give rise to a polymer-enriched region characterized by chains extended normal to the underlying surface [34,38,43,44].

The head groups of PEG 6650-alkyl tail conjugated amphipaths appear readily capable of rearranging to accommodate micelle formation [45], or tail group mediated adsorption at various flat hydrophobic surfaces [44]. In phosphate buffered saline such adsorption appears to saturate, due to the interaction of surface localized PEG molecules, at approximately 0.1 molecules nm^{-2}. The resulting PEG-rich layer is approximately 12 nm thick (i.e., greater than the $\approx$3.5 nm radius of gyration for the PEG in solution) with an average polymer concentration (0.07 g ml^{-1}) much greater than the PEG-rich phase in many two-phase systems [44]. PEG-rich surface regions, such as that described above are able to effectively mask surface charge groups [39,40,46] and reduce the adsorption of many proteins (Refs. [44,46], see also Refs. [2,35–38]) including those which may be involved in mediating the serum half-life of polymer-grafted pharmaceuticals [47]. Distinct polymer-rich interfacial regions may also form at the surfaces of small colloids possessing surface localized PEGs (Refs. [13,45,48], see also Refs. [5–7]).

The PEG–protein partition data in Table 2 lacks specific information related to the topological distribution and conformation of both the grafted PEGs and their protein conjugates. However, knowledge of the behavior of interfacially localized PEG molecules [see above] allows mechanistic speculation on the ability of PEG-grafting to alter both protein surface properties and partition. Such speculation led to the

Table 3
Correlation coefficients (r^2) of Table 1 data

Data and reference	Range of n	Log K vs. n[a] (1st order)	Log K vs. n[a] (2nd order)	Log K vs. PWF[a] (1st order)
PEG 1900–IgG, [15]	0–43	0.956 (4)	0.960 (4)	0.924 (4)
PEG 5000–BSA, [16]	0–46	0.736 (5)	0.870 (5)	0.948 (5)
PEG 5000–BSA, [18]	0–17	0.945 (5)	0.991 (5)	0.990 (5)
PEG 5000–GM-CSF, [21,23]	0–2.5	0.943 (6)	0.945 (6)	0.938 (6)

[a] Correlation coefficient with number of data points in brackets.

data analysis method and results presented below. Consider:

1. Available reactive groups localized at the 'surface' (solution interface) of a protein have a greater tendency to react with functionalized PEGs. Groups located more within a protein's 3-dimensional structure are less reactive with PEG molecules due to, for example, a steric need for PEG or protein molecular reorganization to achieve grafting.

2. Protein amino acid sequence and protein three dimensional structure may help randomize distribution of the most PEG-reactive groups over a protein's surface. This will help randomize the surface distribution of grafted PEGs.

3. Grafting sites are further randomized, especially at low n, by initially grafted PEGs affecting proximally located protein groups. Such hindrance could be steric, or chemical (e.g., grafted PEGs non-covalently interacting with proximal groups so as to decrease their reactivity). These effects might function over a relatively large protein surface area, e.g. 10 nm^2 for PEG 6000 terminally localized on some (non-protein) surfaces [44].

4. As polymer grafting ratio increases the interfacial region of the conjugate will become polymer-enriched, and variously influence the physical and general functional characteristics of the conjugate. General capabilities should be distinguished from specific capabilities such as an enzymatic activity affected by covalent modification of a certain amino acid residue.

5. As polymer grafting increases individual polymer molecules will contribute less to the various properties of the interfacial region, and general alteration of the conjugate's functional characteristics or partition behavior.

6. Data for log K should vary directly with n, but not linearly, as it should reflect how a specific variation in n alters the interfacial properties of the conjugate. The latter will be somewhat unique (analytically sensitive) to each experimental situation but it is expected to decrease non-linearly with grafting. As n increases partition will be more influenced by free energy considerations involving net interaction of the polymer-rich region of the two phases with that of the conjugate. Such considerations will be influenced by many factors, including polymer type and concentration.

7. Graphs of log K versus n should reach a plateau at higher n (and may even decrease).

Potential non-covalent interactions of grafted PEG molecules with protein surface groups suggests, in part, how low level grafting may result in significant surface masking—particularly in small bioactive proteins grafted with PEGs of relatively high molecular mass. It also suggests that similar results might be obtained by (a) greater grafting of lower molecular mass PEGs, or (b) similar grafting to different but proximal protein groups. The generation of specific polymer-enriched regions at higher grafting densities suggests a plausible explanation for other characteristics related to PEG-grafting, e.g., increased resistance to various forms of denaturation. It also explains certain drawbacks such as reduced enzyme activity, in regard to the viscous barrier (to substrate/product diffusion, alteration of protein conformation) imposed by localizing the conjugate in such regions (see Section 1).

If PEG–protein conjugate partition behavior is influenced by the same factors that influence PEG behavior at other surfaces (above refs.) then a number of previously noted but unexplained partition observations are, in fact, expected. For example:

(a) Any model of partitioning which assumes an equal partition contribution from grafted polymer molecules will not hold, however, random the grafting of polymer, particularly at relatively higher values of n.

(b) The best linear fit of log K to n will be provided by proteins derivatized over a small range of n, relative to polymer-to-protein molecular mass ratio.

(c) Due to the many polymer-, phase system- and protein-related factors which influence partition, it should be analytically sensitive to a variety of surface differences in polymer–protein conjugates.

(d) A parameter which more closely reflects non-linear alteration in the conjugate surface with n, should vary more linearly with log K (than n) over a larger range of n and therefore be of more extrapolative value.

Given precise data concerning the partition of various PEG–protein conjugates in a series of well characterized phase systems, plus the surface density, conformation and topographical distribution of their grafted PEGs, it should be possible to define a

accurate (mathematical) model which relates log K to n over a wide range of experimental conditions. In such a case, for any given system and conjugate, log K might be expected to vary with the average surface concentration, e.g., mass of PEG (of given M_r) per unit area [44]. However, given no precise knowledge of such factors, or conjugate geometry, Table 2 data only lends itself to evaluating a simple correlate which is in basic agreement with the above points. This is the weight fraction, i.e. mass ratio, of polymer in the conjugate.

In Table 2 polymer weight fraction (PWF) was expressed according to convention, as $[nM_l \cdot (nM_l + M_p)^{-1}]$ where, M_l and M_p refer to the mean molecular mass of the PEG monomer and protein, respectively [14,15,20,22]. Fig. 3 shows log K versus PWF for the data shown in Figs. 1 and 2 (including the two data points related to PEG 5000–IgG). Fig. 4 exhibits the same data (with a somewhat expanded ordinate scale) in terms of Δ log K versus PWF. Expressing the data and figures in the latter format (a) is easily accomplished, (b) does not contradict conclusions previously based on Figs. 1 and 2, (c) allows better visual comparison of the different experimental data sets, (d) provides a more linear relationship over a larger range of n, especially as regards the BSA data sets discussed above (Table 3) and (e) facilitates comparison among partition data involving greater variation in systems (e.g. native partition), proteins and polymers.

The PWF relationship shown in Fig. 4 makes few assumptions with regard to polymer molecular

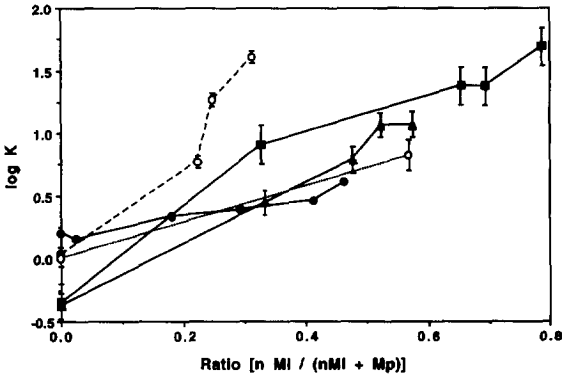

Fig. 3. Log K partition versus polymer weight fraction in PEG–protein conjugate. See Fig. 1 legend.

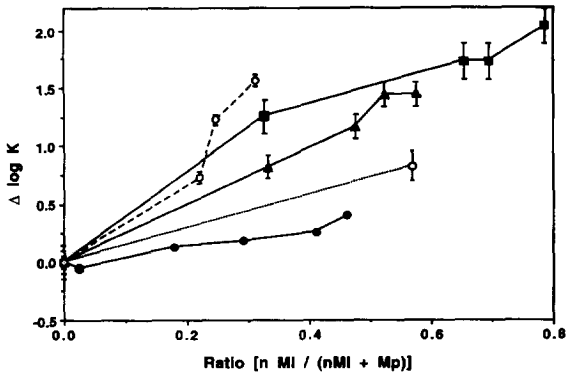

Fig. 4. ΔLog K versus polymer weight fraction in PEG–protein conjugate. See Fig. 1 legend.

weight, branching, linking chemistry, or geometry (in the grafted state). It makes no assumptions about protein type, size, or structure. It is not dependent on data related to polymer inter-chain and intra-chain, or polymer–protein interactions, nor on how the above are affected by grafting topography. Variables which are expected to secondarily affect partition and other conjugate characteristics, and will require more careful study. Figs. 3 and 4 merely confirm that partition is closely related to the average surface density of polymer units in the conjugate. In this they support the ideas given above and, may encourage research to further develop partition as an analytical method for use with polymer–conjugated proteins.

Many questions remain unanswered including the influence of polymer molecular weight, phase system composition and protein (i.e., IgG) type on the partition results described above. Various mathematical modeling approaches can be used to study partition [10–12,22,32] as well as the interaction of neutral polymers at surfaces [33,36–38]. Given more specific data it should be possible to develop and test better models of PEG–protein partition. Such research will lead to a better understanding of the protein surface alterations induced by polymer grafting.

4. Abbreviations

BSA Bovine serum albumin

GM-CSF	Granulocyte–macrophage colony stimulation factor
IgG	Immunoglobulin G:
K	Partition coefficient (concentration ratio of protein in PEG-rich phase and dextran-rich phase)
n	Covalent grafting ratio (moles of PEG per mole of protein):
PEG	Poly(ethylene glycol)
mPEG	Monomethoxypoly(ethylene glycol)
PWF	Polymer weight fraction of PEG in PEG–protein conjugate
r^2	Least squares fit correlation

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References

[1] Delgado, G.E. Francis and D. Fisher, in Critical Reviews in Therapeutic Drug Carrier Systems, CRC Press, Boca Raton, FL, 1992.
[2] J.M. Harris (Editor), Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications, Plenum Press, New York, NY, 1992.
[3] Y. Kodera, T. Sekine, T. Yasukohchi, Y. Kiriu, M. Hiroto, A. Matsushima and Y. Inada, Bioconjugate Chem., 5 (1994) 283.
[4] A. Abuchowski, T. van Es, N.C. Palczuk and F.F. Davis, J. Biol. Chem., 252 (1977) 3578.
[5] M.C. Woodle and D.D. Lasic, Biochim. Biophys. Acta, 1113 (1994) 171.
[6] R. Duncan, Anticancer Drugs, 3 (1992) 175.
[7] D. Fisher, Euro. Pat., 0445131 (1995).
[8] L. Yang and R.W. Murray, Anal. Chem., 66 (1994) 2710.
[9] M. Laliberte, J.-C. Gayet and G. Fortier, Biotechnol. Appl. Biochem., 20 (1994) 397.
[10] H. Walter and G. Johansson (Editors), Aqueous Two-Phase Systems, Methods in Enzymology, Vol. 228, Academic Press, New York, NY, 1994.
[11] P.A. Albertson, Partition of Cell Particles and Macromolecules, Wiley, New York, NY, 2nd ed., 1971, p. 15.
[12] G. Birkenmeir and G. Kopperschläger, 8th International Conference on Partitioning in Aqueous Two-Phase Systems, J. Chromatogr. A, 668 (1994) 1–247.
[13] J.M. Van Alstine, M. Malmsten and D.E. Brooks, Poly(Ethylene Glycol) Amphiphile Adsorption and Liposome Partition, J. Chromatogr. B, 680 (1996) 145.
[14] S.D. Flanagan and S.H. Barondes, J. Biol. Chem., 250 (1975) 1484.
[15] K.A. Sharp, M. Yalpani, S.J. Howard and D.E. Brooks, Anal. Biochem., 154 (1986) 110.
[16] L.J. Karr, S.G. Shafer, J.M. Harris, J.M. Van Alstine and R.S. Snyder, J. Chromatogr., 354 (1986) 269.
[17] L.J. Karr, J.M. Van Alstine, R.S. Snyder, S.G. Shafer and J.M. Harris, J. Chromatogr., 442 (1988) 219.
[18] C. Delgado, J.N. Patel, G.E. Francis and D. Fisher, Biotechnol. Appl. Biochem., 12 (1990) 119.
[19] D.E. Brooks, J.M. Van Alstine, K.A. Sharp and S.J. Stocks, in J.M. Harris (Editor), Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications, Plenum Press, New York, NY, 1992, pp. 57–72.
[20] G. Kopperschlager, Methods Enzymol., 228 (1994) 121–136.
[21] C. Delgado, in R.D. Rogers and M. Eitman (Editors), Aqueous Biphasic Separations: From Metal Ions to Biomolecules, Plenum Press, New York, NY, 1995, pp. 167–176.
[22] D.E. Brooks, K.A. Sharp and D. Fisher, in H. Walter, D.E. Brooks and D. Fisher (Editors), Partitioning in Aqueous Two-Phase Systems, Academic Press, Orlando, FL, 1985, pp. 11–85.
[23] C. Delgado, F. Malik, B. Selisko, D. Fisher and G.E. Francis, J. Biochem. Biophys. Methods, 29 (1994) 237.
[24] L.J. Karr, D.L. Donnelly, A. Kozlowski and J.M. Harris, Methods Enzymol., 228 (1994) 377–390.
[25] S.J. Stocks, A.J. Jones, C.W. Ramey and D.E. Brooks, Anal. Biochem., 154 (1986) 232.
[26] I. Clark-Lewis, A.F. Lopez, L.B. To, M.A. Vadas, J.W. Schrader, L.E. Hood and S.B.H. Kent, J. Immunol., 141 (1988) 881.
[27] T. Peters Jr., Adv. Protein Chem., 37 (1985) 161.
[28] I. Roitt, Essential Immunology, Blackwell Scientific Publications, Oxford, 7th ed., 1991, p. 45.
[29] G.M., Edelman, B.A. Cunningham, W.E. Gell, P.D. Gottlieb, U. Rutishauser and M.J. Waxdal, Proc. Natl. Acad. Sci. USA, 63 (1969) 78.
[30] K. Nilsson and K. Mosbach, Methods Enzymol., 104 (1984) 56.
[31] S. Zalipsky and C. Lee, in J.M. Harris (Editor), Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Applications, Plenum Press, New York, NY, 1992, pp. 347–370.
[32] M. Carlsson, P. Linse and F. Tjerneld, Macromolecules, 26 (1993) 1546.
[33] P. Thiyagarajan, D.J. Chaiko and R.P. Helm Jr., Macromolecules, 28 (1995) 7730.
[34] G.J. Fleer, M.A., Cohen Stuart, J.M.H.M. Scheutjens, T. Cosgrove and B. Vincent, Polymers at Interfaces, Chapman and Hall, London, 1993.

[35] K.L. Prime and G.W. Whitesides, J. Am. Chem. Soc., 115 (1993) 10 714.
[36] Y.S. Lin, V. Hlady and C.-G. Gölander, Colloids Surf. B, 3 (1994) 49.
[37] S.I. Jeon, J.H. Lee, J.D. Andrade and P.G. De Gennes, J. Colloid Interf. Sci., 142 (1991) 149 and 159.
[38] H. Hommel, A. Halli, A. Touhami and A.P. Legrand, Colloids Surf. A, 111 (1996) 67.
[39] N.L. Burns, J.M. Van Alstine and J.M. Harris, Langmuir, 11 (1995) 2768.
[40] K. Emoto, J.M. Harris and J.M. Van Alstine, Anal. Chem., (1996) in press.
[41] C. Karlstrom, J. Phys. Chem., 89 (1985) 4962.
[42] P.M. Claesson, R. Kjellander, P. Stenius and H.K. Christenson, J. Chem. Soc. Faraday Trans., 1, 82 (1986) 2735.
[43] M.A. Cohen-Stuart, F.H.W.H. Waajen, T. Cosgrove, B. Vincent and T. Crowley, Macromolecules, 17 (1984) 1825.
[44] M. Malmsten and J.M. Van Alstine, J. Colloid Interf. Sci., 177 (1996) 502.
[45] J.M. Van Alstine, K.A. Sharp and D.E. Brooks, Colloids Surf., 17 (1983) 115.
[46] E. Österberg, K Bergström, K. Holmberg, T.P. Schuman, J.A. Riggs, N.L. Burns, J.M. Van Alstine and J.M. Harris, J. Biomed. Materials Res., 29 (1995) 741.
[47] M. Malmsten, B. Lassen, J.M. Van Alstine and U.R. Nilsson, J. Colloid Interf. Sci., 178 (1996) 123.
[48] M. Yokoyama, T. Okano, Y. Sakurai, K. Kataoka, J. Control. Release, 32 (1994) 269.