# Dynamic and Electrophoretic Light Scattering of a Water-Soluble Complex Formed between Pepsin and Poly(ethylene glycol)

**Jiulin Xia**† **and Paul L. Dubin**

*Department of Chemistry, Indiana–Purdue University, Indianapolis, Indiana 46223*

**Etsuo Kokufuta***

*Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305, Japan*

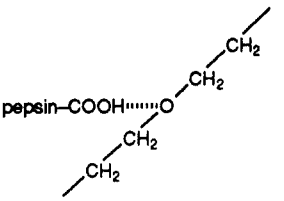
*Received March 1, 1993*

*Revised Manuscript Received September 13, 1993*

The complexation of proteins with water-soluble synthetic polymers in an aqueous system is interesting from two points of view.[1] The first concerns the way in which the polymers interact with nonflexible protein molecules through electrostatic, hydrogen-bonding, and hydrophobic interactions, an understanding of which could provide a better explanation of the mechanisms of macromolecular interaction available in nature. The second concerns the extent to which biochemical activity is maintained in the resulting complexes, the answer to which is central to the molecular design of composite protein/polymer systems, such as immobilized enzymes, as well as the process design for protein separation using water-soluble polymers.

A number of studies have dealt with complexes formed between polyelectrolytes and proteins under different conditions of pH and salt concentration.[2-4] The turbidimetric measurements primarily employed in previous studies[2-4] could easily monitor the resulting water-insoluble complexes, the form of which was either a complex coacervate or an amorphous precipitate. Under special pH and salt concentration conditions, the formation of "seemingly" soluble complexes has been observed in several systems consisting of proteins and polyelectrolytes.[5-9] On the basis of quasi-elastic light scattering (QELS) and electrophoretic light scattering (ELS) techniques, it has been reported that several species (presumably composed of an intrapolymer complex and its aggregates) with different Stokes diameters were formed when the pH of the protein solutions was increased in the presence of polycations such as poly(diallyldimethylammonium chloride).[5,6] However, the observed size distribution of these species does not conclusively establish whether, during the initial stage of the complexation, several protein molecules are bundled together by a polyion to form intrapolymer complexes which then subsequently aggregate. In some respects, it is preferable at present to study molecular complex formation using a system consisting of fully soluble complexes in which no aggregation occurs.

One of the authors has recently demonstrated that pepsin forms a water-soluble complex with poly(ethylene glycol) (PEG) at pH 3.0, but not at pH 4.5, presumably through the hydrogen bonding of the protein–COOH groups with the ether groups in PEG:[10]




Neither precipitation nor turbidity appeared in this system after the mixing of the protein and polymer at different ratios. This is clearly different from protein/polyelectrolyte systems which display turbidity to a greater or lesser degree at conditions corresponding to strong interaction. We have therefore tried to measure the properties of such complexes using QELS and ELS techniques.

The porcine pepsin (a typical acid gastric protease) used here was identical to the sample used in the previous study.[10] The absolute molecular weight of pepsin was 34 542, as determined by its amino acid sequence. PEG with $M_w = 1.4 \times 10^4$ was purchased from Aldrich Chemical Co. Since the amino acid sequence of pepsin has been reported previously,[11,12] the complexation was studied at pHs 3.0 and 4.5 under different molar ratios ($R_m$) of the ether groups in PEG to all of the pepsin acidic groups (43 carboxyl and 16 phenolic OH).[13] The samples were prepared by mixing the desired amounts of dust-free stock solutions of PEG and pepsin, each of which was obtained by filtration using a Gelman 0.2-$\mu$m syringe filter, to yield a final pepsin concentration of about 1 mg/mL, with the PEG concentration depending on $R_m$. The pH of each sample was then adjusted with a very small amount of 0.1 M HCl. Pure water was used as the solvent in the preparation of both stock solutions.

QELS measurements were carried out at scattering angles from 30° to 150° using a Brookhaven system (Holtsville, NY) equipped with a 72-channel digital autocorrelator (BI-2030 AT) and a Jodon 15-mW He–Ne laser (Ann Arbor, MI). We obtained the homodyne intensity–intensity correlation function $G(q,t)$, with $q$ (the amplitude of the scattering vector) given by $q = (4\pi\tilde{n}/\lambda) \sin(\theta/2)$, where $\tilde{n}$ is the refractive index of the medium, $\lambda$ is the wavelength of the excitation light in a vacuum, and $\theta$ is the scattering angle. $G(q,t)$ is related to the time correlation function of concentration fluctuations $g(q,t)$ by $G(q,t) = A[1 + bg(q,t)^2]$, where $A$ is the experimental base line and $b$ is the fraction of the scattered intensity arising from concentration fluctuations. The quality of the measurements was verified by determining that the difference between the measured and calculated $A$ values was less than 1%. The mean decay constant $\Gamma$ was obtained by the method of cumulants and used to calculate the apparent diffusion coefficient $D_{eff}$ ($=\Gamma/q^2$). The apparent Stokes diameter, $D_s$, was then obtained using $D_s = kT/6\pi\eta D_{eff}$, where $k$ is the Boltzman constant, $T$ is the absolute temperature, and $\eta$ is the viscosity of the solvent.

ELS measurements were made at a fixed scattering angle of 25.6° using a Coulter DELSA 400 apparatus (Hialeah, FL). The electric field was applied at a constant current of 0.3 mA.

Neither precipitation nor turbidity appeared in any of the samples examined. The $D_s$ values obtained from QELS via the method of cumulants are shown in Table I, together with the electrophoretic mobilities, $u$, determined by ELS and also with the results[10] of previous viscometric measurements. Our samples were assumed to be multicomponent systems of PEG, pepsin, and the complex of these two in a state of equilibrium. Therefore, the observed $D_s$

† Current address: Life Technologies, Inc., P.O. Box 6009, Gaithersburg, MD 20884-9980.

0024-9297/93/2226-6688$04.00/0 

**Table I. Apparent Stokes Diameters ($D_s$), Electrophoretic Mobilities ($u$), and Reduced Viscosities ($\eta_{sp}/C_{pepsin}$) Obtained from Aqueous Pepsin/PEG Systems at pHs 3.0 and 4.5 as a Function of the Mixing Ratio ($R_m$) of the Polymer and Protein**

| | $D_s$ (nm) | | | | $\eta_{sp}/C_{pepsin}$ (dL/g)[b] | |
|---|---|---|---|---|---|---|
| | | | $u$ ($\mu$m·cm/V·s) | | | |
| $R_m$[a] | pH 3.0 | pH 4.5 | pH 3.0 | pH 4.5 | pH 3.0 | pH 4.5 |
| 0[c] | 5 | 5 | 0.08 ± 0.02 | −1.26 ± 0.02 | | |
| 1 | 7 | 7 | 0.04 ± 0.02 | −1.26 ± 0.02,[d] 0[e] | 0.071 | 0.073 |
| 5 | 8 | 7 | 0.06 ± 0.02 | −1.27 ± 0.02,[d] 0[e] | 0.082 | 0.068 |
| 20 | 9 | 6 | 0.06 ● 0.02 | −1.28 ● 0.02,[d] 0[e] | 0.089 | 0.074 |
| 50 | 16 | 7 | 0.04 ± 0.02 | −1.25 ± 0.02,[d] 0[e] | 0.096 | 0.072 |
| 100 | 27 | 7 | 0.04 ± 0.02 | −1.24 ± 0.02,[d] 0[e] | 0.106 | 0.073 |
| 150 | 32 | | see footnote *f* | | 0.109 | 0.073 |
| 200 | 31 | | see footnote *f* | | 0.110 | 0.072 |
| ∞[g] | 6 | 6 | 0 | 0 | | |

[a] Weight and molar base ratios of PEG to pepsin can be calculated from $R_m$ using equations in ref 13. [b] Data for $R_m$ = 0–150 were cited from a previous study[10] with that for $R_m$ = 200 newly measured for this study; in both, the values of $\eta_{sp}/C_{pepsin}$ were determined by viscometric measurements of aqueous pepsin solutions including different amounts of PEG ($M_w$ = 1.98 × 10⁴) at 25 °C using the relation $\eta_{sp}/C_{pepsin} = \{(\eta/\eta_0) - 1\}/C_{pepsin}$, where $\eta$ is the viscosity of the sample containing both pepsin and PEG and $\eta_0$ is the viscosity of the solvent including PEG only. [c] Determined from uncomplexed pepsin (i.e., PEG-free pepsin solution). [d] Corresponds to pepsin molecules. [e] Corresponds to PEG molecules. [f] Mobilities could not be determined from the electrophoretic diagrams obtained at $R_m \geq 150$ due to incomplete resolution of a broad peak with a shoulder, although the positions of the peak and shoulder were close to $u$ = 0 $\mu$m·cm/V·s (excess and uncomplexed PEG) and to $u$ = 0.03–0.05 $\mu$m·cm/V·s (presumably a pepsin/PEG complex), respectively. [g] Determined from uncomplexed PEG (i.e., pepsin-free PEG solution).

may not simply correspond to the size of the complex, because the diffusion mode detected presumably contains contributions from all scattering species. However, $D_s$ at pH 3.0 increases with an increase in $R_m$ and reaches a constant value at $R_m \geq 150$, whereas $D_s$ at pH 4.5 is independent of $R_m$ within the accuracy of QELS. This last result also shows that the contribution of free PEG to the scattering signal is essentially negligible. Samples at pHs 3.0 and 4.5 with the same $R_m$ should contain the same amounts of the polymer and protein; therefore, the observed $R_m$ dependence of $D_s$ strongly suggests that the $D_s$ values at pH 3.0 primarily reflect contributions due to the complex. As a result, it can be said that pepsin and PEG form a water-soluble complex at pH 3.0, but not at pH 4.5. This is in agreement with the results of viscometric measurements; that is, the viscosity increased with the addition of PEG at pH 3.0 but remained unchanged at pH 4.5, such viscometric changes being assumed to indicate the formation of a water-soluble pepsin/PEG complex at pH 3.0 (see ref 10).

ELS at pH 4.5 reveals two species with $u$ = 0 $\mu$m·cm/V·s (PEG) and −1.28 ± 0.02 to −1.24 ± 0.02 $\mu$m·cm/V·s (pepsin); from this result it would appear that the mobilities of both species are independent of $R_m$. At pH 3.0, only one peak was detected at $R_m \leq 100$ (see footnote *f* in Table I). However, we can observe a small but statistically significant decrease in $u$ as PEG is added to the protein solution. This can be explained as follows. The electrophoretic velocity ($v$) of charged particles suspended in a viscous medium under an electrical potential ($X$) is proportional to the electrical charge ($Q$) of the particles and inversely proportional to the frictional coefficient ($f$): i.e., $v \propto (Q/f)X$, where in pure water $f = 6\pi\eta R_s$ ($\eta$, viscosity of solvent; $Rs$, radius of a hydrodynamically equivalent sphere), according to Stokes' law. Since the complexation at pH 3.0 is thought to occur through hydrogen bonding of the

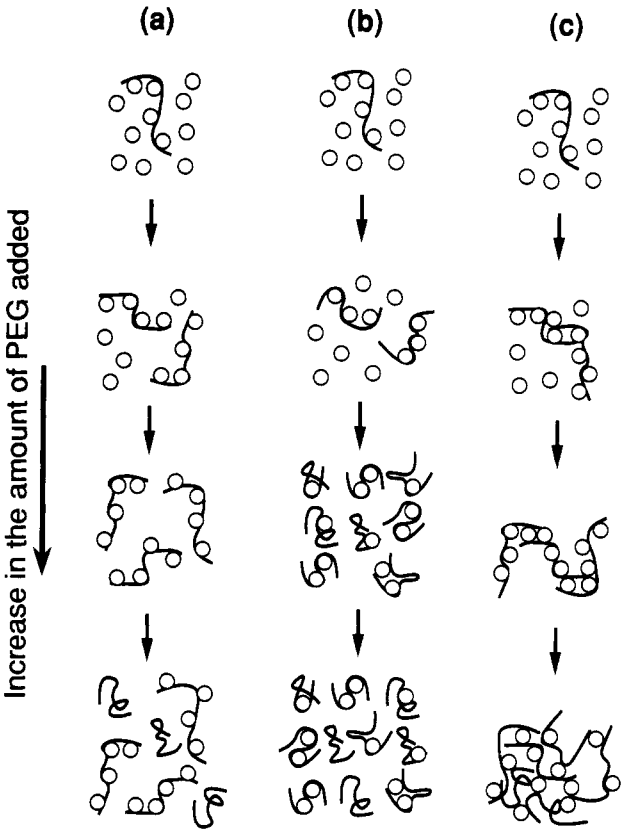



**Figure 1.** Schematic illustration for complexation of PEG with pepsin.

protein–COOH groups with the ether groups in PEG, the electrical charges due to the five positive ions (i.e., charged two amino, one imidazolyl, and two guanidyl groups) of pepsin remain unaltered before and after the complexation. Thus, the complexation could result in a decrease in the mobility through an increase in $f$ due to the neutral PEG chain(s) bound to the positively charged proteins.

It is certain that pepsin and PEG form a water-soluble complex at pH 3.0; we will now discuss the mechanism of this complexation. There seem to be three possibilities with respect to the complexation between pepsin and PEG (see Figure 1): In a, where we assume cooperative binding of protein to protein, the number of proteins bound per polymer ($n$) is constant; b shows the anticooperative case, where $n$ must decrease as $R_m$ increases; c illustrates "interpolymer" complexation. Since the observed $D_s$ values are the averages from the complex and the free proteins (as shown above, the scattering contribution of free PEG is negligible), complexation according to mechanism a causes a gradual increase in $D_s$ until all the free proteins are consumed. For b, $D_s$ should at first increase but must eventually decrease, as the addition of more PEG only makes $n$ smaller. Mechanism c is not consistent with the limiting value of $D_s$ attained at high $R_m$. Thus, only mechanism a seems to explain our QELS results in Table I. Because the equilibrium constant for complexation between PEG and pepsin is not large, $D_s$ reaches a limiting value between $R_m$ = 100 and 150 (corresponding to molar ratios of PEG to pepsin of 19 and 28; see ref 13). At present, we cannot explain the mechanism for the cooperative binding of the proteins to PEG. More detailed studies will be required to resolve the contributions of complex and free protein to the apparent Stokes diameter.

The main conclusions to be drawn from this study are as follows: (a) QELS helps us to observe complex formation between protein and nonionic polymer; (b) the effect of pH suggests that hydrogen bonds are involved in com-

plexation; and (c) the behavior of $D_s$ is consistent with somewhat cooperative binding of pepsin to PEG in an intrapolymer complex, which has expanded dimensions due to interprotein steric and coulombic repulsions.

**Acknowledgment.** We wish to thank Dr. Y. Kim and Eli Lilly and Co. for the use of electrophoretic light scattering instrumentation. This research was supported in part in Japan by grants from the Ministry of Education (No. 05044077) and the New Energy and Industrial Technology Development Organization (NEDO) and in the United States by grants from the National Science Foundation (NSF-DMR 9014945), American Chemical Society (ACS-PRF No. 2532-AC7B), Eli Lilly and Co., and the Exxon Education Research Fund.

## References and Notes

(1) Kokufuta, E. In *Macromolecular Complexes in Chemistry and Biology*; Dubin, L., Davis, R., Thies, C., Bock, J., Schulz, D., Eds.; Springer-Verlag: Heidelberg, Germany, 1993; in press.
(2) Park, J. M.; Muhoberac, B. B.; Dubin, P. L.; Xia, J. *Macromolecules* **1992**, *25*, 290 and references therein.
(3) Kokufuta, E.; Shimizu, H.; Nakamura, I. *Macromolecules* **1982**, *15*, 1616.
(4) Kokufuta, E.; Takahashi, K. *Polymer* **1990**, *31*, 1177 and references therein.
(5) Dubin, P. L.; Murrell, J. M. *Macromolecules* **1988**, *21*, 2291.
(6) Xia, J.; Dubin, P. L.; Muhoberac, B. B.; Kim, Y. *ACS Abstr. Paper* **1992**, *203*, 362-COLL.
(7) Ruckpaul, K.; Rein, H.; Jänig, G.-R.; Pfeil, W.; Ristau, O.; Damaschun, B.; Damaschum, H.; Müller, J.-J.; Pürschel, H.-V.; Belke, J.; Scheler, W. *Stud. Biophys.* **1972**, *34*, 81.
(8) Amiconi, G.; Zolla, L.; Vecchini, P.; Brunori, M.; Antonini, E. *Eur. J. Biochem.* **1977**, *76*, 336.
(9) Kabanov, V. A.; Zezin, A. B.; Mustafaev, M. I.; Kasaikin, V. A. In *Polymeric Amines and Ammonium Salts*; Goethals, E. J., Ed.; Pergamon: New York, 1980; p 173.
(10) Kokufuta, E.; Nishimura, H. *Polym. Bull.* **1991**, *26*, 277.
(11) Tang, J.; Sepulveda, P.; Marciniszyn, J., Jr.; Chen, K.; Huang, W.-Y.; Tao, N.; Liu, D.; Lanier, J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 3437.
(12) Sepulveda, P.; Marciniszyn, J., Jr.; Liu, D.; Tang, J. *J. Biol. Chem.* **1975**, *250*, 5082.
(13) $R_m$ can be related to the weight ratio ($R_w$) of PEG to pepsin by $R_w = 0.0752R_m$, because the acidic group content for pepsin = $1.71 \times 10^{-3}$ mol/g and the molecular weight of the repeating units for PEG = 44. Also, we can relate $R_m$ to the molar ratio ($R_m'$) of PEG to pepsin by $R_m' = 0.185R_m$ using the molecular weights.