# Diffusion and Release of Solutes in Pluronic-g-poly(acrylic acid) Hydrogels

**ARTICLE** *in* LANGMUIR · OCTOBER 2003

Impact Factor: 4.46 · DOI: 10.1021/la034822w



**3 AUTHORS:**

John Cleary
Institute of Technology, Carlow
**39** PUBLICATIONS **274** CITATIONS
SEE PROFILE

Lev Bromberg
Massachusetts Institute of Technology
**147** PUBLICATIONS **4,399** CITATIONS
SEE PROFILE

Edmond Magner
University of Limerick
**96** PUBLICATIONS **2,583** CITATIONS
SEE PROFILE

# Diffusion and Release of Solutes in Pluronic-*g*-poly(acrylic acid) Hydrogels

John Cleary,[†] Lev E. Bromberg,[‡] and Edmond Magner*,[†]

*Materials and Surface Science Institute and Department of Chemical and Environmental Sciences, University of Limerick, Plassey, Co. Limerick, Ireland, and Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

*Received May 13, 2003*

Pluronic−PAA, a thermogelling copolymer composed of side chains of poly(acrylic acid) (PAA) grafted onto a backbone of Pluronic copolymer, is of interest as a vehicle for the controlled release of compounds. The diffusion of various electrochemically active solutes (ferricyanide, the redox protein cytochrome *c*, and a range of ferrocene derivatives) with varying sizes and hydrophobicities has been examined in solutions and hydrogels of Pluronic−PAA. Release of the solutes from the hydrogel into aqueous media has also been examined. Small hydrophilic molecules diffused freely through the hydrogel, but diffusion of more hydrophobic species was retarded due to their association with hydrophobic micellar aggregates formed on gelation of Pluronic−PAA. The rate of release of solutes from the hydrogel into aqueous media also decreased with increasing hydrophobicity. Constant-rate release was observed over a considerable time period for hydrophobic materials. Cytochrome *c* exhibited reduced diffusion coefficients at elevated temperatures due to obstruction by the hydrogel network. Release of the protein from the hydrogel was significantly affected by ionic strength, suggesting that electrostatic interactions were an important factor in the mobility of cytochrome *c* in Pluronic−PAA hydrogels.

## Introduction

Triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) with the structure PEO−PPO−PEO are known generically as poloxamers and under the tradenames Pluronic or Synperonic. Aqueous solutions of these materials undergo marked changes in viscosity due to the formation of micelles which pack in a cubic lattice to form a gel-like medium within certain concentration and temperature limits.[1−8] The structure of PEO−PPO−PEO micelles in aqueous solutions is often described by a core−corona model in which a spherical core composed of PPO segments is surrounded by a corona composed of the more hydrated PEO segments.[9−11] When tightly packed, this structure yields a cubic lattice capable of solubilizing hydrophobic compounds within the micellar interior. This has led to a number of applications including sustained release of perfume compounds,[12,13] separation technology,[14−16] and controlled drug delivery.[17−21] Pluronic F127 has been reported to be among the least toxic of the commercially available Pluronics[22] and hence has been used extensively in drug delivery studies.[23−26] Pluronic F127 has the composition $EO_{100}PO_{65}EO_{100}$ with an average molecular weight (MW) of 12 600 Da. The MW of the propylene oxide (PO) segment is 3780 Da, and the ethylene oxide (EO) content is 70 wt %.[9]

Bromberg and co-workers have prepared and characterized graft copolymers of Pluronic block copolymers and poly(acrylic acid) (PAA).[9,27−44] Briefly, the synthesis

* To whom correspondence should be addressed. E-mail: edmond.magner@ul.ie. Phone: (353) 61 202629. Fax: (353) 61 213529.

[†] University of Limerick.

[‡] Massachusetts Institute of Technology.

(1) Chen-Chow, P. C.; Frank, S. G. *Int. J. Pharm*. **1981**, *8*, 89−99.
(2) Gilbert, J. C.; Hadgraft, J.; Bye, A.; Brookes, L. G. *Int. J. Pharm*. **1986**, *32*, 223−228.
(3) Chi, S. C.; Jun, H. W. *J. Pharm Sci*. **1991**, *8*, 280−283.
(4) Juhasz, J.; Lenaerts, V.; Raymond, P.; Ong, H. *Biomaterials* **1989**, *1*, 265−268.
(5) Wu, C.; Liu, T.; Chu, B. *Electrophoresis* 1**998**, *19*, 231−241.
(6) Liang, D.; Chu, B. *Electrophoresis* **1998**, *19*, 2447−2453.
(7) Wu, C.; Liu, T.; Chu, B.; Schneider, D. K.; Graziano, V. *Macromolecules* **1997**, *30*, 4574−4583.
(8) Hadden, D. A.; Rill, R. L.; McFaddan, L.; Locke, B. R. *Macromolecules* **2000**, *33*, 4235−4248.
(9) Bromberg, L. E.; Ron, E. S. *Adv. Drug Delivery Rev*. **1998**, *31*, 197−221.
(10) Alexandridis, P.; Hatton, T. A. *Colloids Surf., A* **1995**, *96*, 1−46.
(11) Goldmints, I.; Yu, G.; Booth, C.; Smith, K. A.; Hatton, T. A. *Langmuir* **1999**, *15*, 1651−1656.
(12) Saito, Y.; Miura, K.; Tokuoka, Y.; Kondo, Y.; Abe, M.; Sato, T. *J. Dispersion Sci. Technol*. **1996**, *17*, 567−576.
(13) Suzuki, K.; Saito, Y.; Tokuoka, Y.; Abe, M.; Sato, T. *J. Am. Oil Chem. Soc*. **1997**, *74*, 55−59.
(14) Rill, R. L.; Liu, Y.; Van Winkle, D. H.; Locke, B. R. *J. Chromatogr., A* **1998**, *817*, 287−295.
(15) Choi, Y.-K.; Lee, S.-B.; Lee, D.-L.; Ishigami, Y.; Kajiuchi, T. *J. Membr. Sci*. **1998**, *148*, 185−194.
(16) Miksik, I.; Deyl, Z.; Kasicka, V. *J. Chromatogr., B* **2000**, *741*, 37−42.
(17) Desai, S. D.; Blanchard, *J. Pharm. Sci*. **1998**, *87*, 226−230.
(18) Rapaport, N. *Colloids Surf., B* **1999**, *16*, 93−111.
(19) Moore, T.; Croy, S.; Mallapragada, S.; Pandit, N. *J. Controlled Release* **2000**, *67*, 191−202.
(20) Lin, H.-R.; Sung, K. C. *J. Controlled Release* **2000**, *69*, 379−388.
(21) Kabanov, A. V.; Batrakova, E. V.; Alakhov, V. Y. *Adv. Drug Delivery Rev*. **2002**, *54*, 759−779.
(22) Johnston, T. P.; Miller, S. C. *J. Parenter. Sci. Technol*. **1985**, *39*, 83−88.
(23) Guzman, M.; Garcia, F. F.; Molpeceres, J.; Abeturas, M. *Int. J. Pharm*. **1992**, *80*, 119−127.
(24) Gilbert, J. C.; Whiteman, M. *J. Pharm. Pharmacol. Suppl*. **1990**, *42*, 128.
(25) Gilbert, J. C.; Richardson, J. L.; Davies, M. C.; Palin, K. J.; Hadgraft, J. *J. Controlled Release* **1987**, *5*, 113−118.
(26) Futs, K. A.; Johnston, T. P. *J. Parenter. Sci. Technol*. **1990**, *44*, 58−65.
(27) Bromberg, L.; Orkisz, M.; Roos, E.; Ron, E. S.; Schiller, M. *J. Controlled Release* **1997**, *48*, 305−308.
(28) Bromberg, L. E.; Mendum, T. H. E.; Orkisz, M. J.; Ron, E. S.; Lupton, E. S. *Proc. Polym. Mater. Sci. Eng*. **1997**, *76*, 273−275.
(29) Orkisz, M. J.; Bromberg, L.; Pike, R.; Lupton, E. C.; Ron, E. S. *Proc. Polym. Mater. Sci. Eng*. **1997**, *76*, 276−277.
(30) Bromberg, L. E.; Orkisz, M. J.; Ron, E. S. *Polym. Prepr*. (*Am. Chem. Soc., Div. Polym. Chem*.) **1997**, *38*, 626−627.
(31) Bromberg, L. *Langmuir* **1998**, *14*, 5806−5812.
(32) Bromberg, L. *Macromolecules* **1998**, *31*, 6148−6156.
(33) Bromberg, L. *J. Phys. Chem. B* **1998**, *102*, 1956−1963.
(34) Bromberg, L. *J. Phys. Chem. B* **1998**, *102*, 10736−10744.

10.1021/la034822w CCC: $25.00 
Published on Web 09/30/2003

involves free-radical polymerization of acrylic acid (AA) with chain transfer to the Pluronic resulting in grafting of PAA chains onto the Pluronic backbone.[34] In this study, Pluronic F127 was chosen as a component of the Pluronic−PAA copolymer. The most striking feature of Pluronic−PAA is the ability of its aqueous solutions to form a gel at body temperatures, without phase separation, at low polymer concentrations. At low temperatures, the viscosity of 1−3 % (w/w) aqueous solutions of Pluronic−PAA does not vary significantly. At temperatures above 20 °C, a rapid 10−$10^3$-fold increase in viscosity occurs over a range of several degrees in semidilute solutions of the Pluronic−PAA.[29]

The gelation of the Pluronic−PAA solutions is believed to be phenomenologically analogous to the formation of gel phases in concentrated Pluronic solutions.[9] Dynamic light scattering experiments have shown that the increase of the intensity of the scattered light at temperatures is almost coincidental with the gelation of Pluronic−PAA solutions.[45] The onset of gelation coincides with the formation of uniformly spaced micellar aggregates above a certain critical micellization temperature (cmt). These micelle-like aggregates are formed by entropy-driven aggregation of the PPO segments and act as physical cross-linkers for gelation.[46] Small-angle neutron scattering (SANS) analysis has shown that the aggregates consist of a spherical core of dehydrated PPO segments, surrounded by an "inner corona" of more hydrated PEO segments and an "outer corona" of ionized PAA segments. The radii of the core and inner corona have been measured as 30 and 60 Å, respectively, for a 1% Pluronic (F127)−PAA gel.[38]

A study on the kinetics of the in vitro release of steroid hormones from Pluronic−PAA hydrogels[28] suggested that the rate of release was bimodal. The fast mode was associated with those hormone molecules that were trapped in the PAA corona and not solubilized in the micelles. The second, much slower mode was associated with diffusion of molecules from the hydrophobic cores of the Pluronic−PAA aggregates. A study of the rate of release of two hydrophobic fluorescent probes, pyrene and estradiol, from Pluronic−PAA gels[41] confirmed the presence of slow and fast modes. The fast mode was a result of "burst" release of 5−11% of the total amount of probe within 1 min. Under physiological conditions (37 °C, pH 7.4), the slow release mode was characterized by diffusion coefficients of the order of $10^{-14}$ $cm^2/s$ and was ascribed to diffusion of the probes out from the core of the micellar aggregates. The diffusion of various water-soluble solutes in Pluronic−PAA systems using the fluorescence recovery after photobleaching (FRAP) technique was explored[46] and showed that the diffusional behavior of small solutes depended on their hydrophobicity and extent of partitioning into the micellar aggregates when gelation occurred. The diffusion coefficient of sodium fluorescein (Na-Fl) was shown to decrease slightly in the gelation transition region of a 2% (w/w) Pluronic−PAA solution, but at higher temperatures the diffusion coefficient ($D$) reverted back to its original value, suggesting that the dye was not immobilized within the micellar cores. The transient decrease in $D_{\text{Na-Fl}}$ was attributed to changing interactions of the dye with the PEO or PPO segments of the copolymer. The diffusion coefficient of a more hydrophobic dye, fluorescein diacetate (Fl-DA), also decreased significantly over the temperature range coinciding with the gelation transition region of the copolymer solution, but $D_{\text{Fl-DA}}$ did not increase significantly at higher temperatures. This indicated that the more hydrophobic dye was solubilized in the micellar interior. Log $P$ values (where $P$ is the octanol/water partition coefficient) for Fl-DA and Na-Fl were 3.51 and −0.67, respectively.

Proteins were shown to be restricted in their motion through the gels, with the relative diffusion coefficients of bovine serum albumin (BSA) and concanavalin A undergoing significant decreases coinciding with increasing viscosity in 2 wt % Pluronic−PAA solutions.[46] The decrease in protein diffusion rate was attributed not to partition into hydrophobic micellar cores but rather to hindered diffusion effects as the proteins negotiated a more torturous path through the structured gel. This effect was described using an obstruction model.[46] Dextrans, which are flexible polymers, were unaffected by the structures formed in Pluronic−PAA gels.[46] The diffusion of dextrans followed predictions of the Zimm model for flexible random coil polymers, consistent with the radius of gyration of the dextrans used being much smaller than the effective mean pore size in Pluronic−PAA gels.

In the present work, we examine the diffusion of various solute molecules in Pluronic−PAA systems using electrochemical methods. Redox probes of varying size and character were used to examine the effect of Pluronic−PAA solutions and gels on solute diffusion rates. The probes used were ferricyanide, acetylferrocene (AcFc), ferrocene derivatives with alcohol-bearing alkyl side chains of length 10, 14, and 18 carbon atoms (denoted $FcOHC_x$ with $x$ = 10, 14, and 18, respectively), and cytochrome *c*, a redox protein with a MW of 12 400 Da whose electrochemistry has been well characterized.[47−53] The rate of release of solutes from Pluronic−PAA hydrogels into aqueous solutions has also been examined. Diffusion and release profiles were found to be highly dependent on the nature of the solute involved, and the mechanisms of diffusion in Pluronic−PAA solutions and hydrogels are discussed.

## Experimental Section

**Materials.** The following chemicals were obtained from Sigma-Aldrich: nitric acid (70%), poly(acrylic acid) (average MW, ca. 2000), sodium chloride (99.5%), sodium phosphate (99%), potas-

(35) Bromberg, L. *Ind. Eng. Chem. Res.* **1998**, *37*, 4267−4274.
(36) Bromberg, L. E.; Goldfeld, M. G. *Polym. Prepr.* (*Am. Chem. Soc., Div. Polym. Chem.*) **1998**, *39*, 681−682.
(37) Bromberg, L. *Polym. Prepr.* (*Am. Chem. Soc., Div. Polym. Chem.*) **1999**, *40*, 616.
(38) Huibers, P. D. T.; Bromberg, L. E.; Robinson, B. H.; Hatton, T. *Macromolecules* **1999**, *32*, 4889−4894.
(39) Bromberg, L. E.; Barr, D. P. *Macromolecules* **1999**, *32*, 3649−3657.
(40) Bromberg, L.; Salvati, L. *Bioconjugate Chem.* **1999**, *10*, 678−686.
(41) Bromberg, L.; Magner, E. *Langmuir* **1999**, *15*, 6792−6798.
(42) Bromberg, L.; Temchenko, M. *Langmuir* **1999**, *15*, 8627−8632.
(43) Bromberg, L.; Temchenko, M.; Colby, R. H. *Langmuir* **2000**, *16*, 2609−2614.
(44) Ron, E. S.; Bromberg, L.; Luszak, S.; Kearney, M.; Deaver, D. R.; Schiller, M. *Proc. Int. Symp. Controlled Release Bioact. Mater.* **1997**, *24*, 407−408.
(45) Bromberg, L.; Orkisz, E.; Roos, E.; Ron, E. S.; Schiller, M. *Proceedings of the Fourth European Symposium on Controlled Release*, Noordwijk aan Zee, Netherlands, 1996; pp 126−131.
(46) Ho, A. K.; Bromberg, L. E.; O'Connor, A. J.; Perera, J. M.; Stevens, G. W.; Hatton, T. A. *Langmuir* **2001**, *17*, 3538−3544.
(47) Albery, W. J.; Eddowes, M. J.; Hill, H. A. O.; Hillman, A. R. *J. Am. Chem. Soc.* **1981**, *103*, 3904−3910.
(48) Armstrong, F. A.; Hill, H. A. O.; Walton, N. J. *Acc. Chem. Res.* **1988**, *21*, 407−413.
(49) Bond, A. M.; Hill, H. A. O.; Komorsky-Lovric, S.; Lovric, M.; McCarthy M.; Psalti, I. S. M.; Walton, N. J. *J. Phys. Chem.* **1992**, *96*, 8100−8105.
(50) Xie, Y.; Dong, S. *Bioelectrochem. Bioenerg.* **1992**, *29*, 71−79.
(51) Armstrong, F. A.; Bond, A. M.; Hill, H. A. O.; Oliver, B. N.; Psalti, I. S. M. *J. Am. Chem. Soc.* **1989**, *111*, 9185−9189.
(52) Szucs, A.; Novak, M. *J. Electroanal. Chem.* **1995**, *383*, 75−84.
(53) Szucs, A.; Novak, M. *J. Electroanal. Chem.* **1995**, *384*, 47−55.

sium ferricyanide (99%), 1-bromooctane (99%), 1-bromohexadecane (97%), acetylferrocene (95%), silica gel 60, 4-mercaptopyridine (95%), cytochrome *c* from horse heart (95%), fluoresceinamine (isomer I, 98%), and 1-[(3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (98+%). 4,4′-Bipyridyl (99%) and 1-bromododecane (97%) were obtained from Fluka. Magnesium turnings (99.5+%) and diethyl ether dried (maximum of 0.0075% $H_2O$) were obtained from Riedel-de Haen. Poly(ethylene oxide) (MW 3400) was obtained from Polysciences, Inc., and hydrochloric acid (37%) was obtained from Merck. Ethyl acetate and petroleum ether were obtained from BDH Laboratory Supplies. Alumina (1 $\mu$m) was obtained from BAS. Spectra/Por cellulose ester membrane (molecular weight cutoff, 10 000 Da) was obtained from Spectrum, Laguna Hills, CA. All chemicals were of the highest purity available and were used as received.

Purified water (18.2 MΩ) was obtained from an Elgastat Maxima water purification system. Electrochemical measurements were carried out using Autolab (PGSTAT 10) and CH Instruments (CHI-802) potentiostats. Gold and platinum working electrodes (1.6 mm diameter), Ag/AgCl reference electrodes, and Pt wire counter electrodes were obtained from BAS and IJ Cambria Scientific. Temperature control was achieved by immersing the electrochemical cell in a water bath with a Lauda E100 temperature controller. NMR spectra were obtained using a JEOL FX90Q Fourier transform NMR spectrometer. Fourier transform infrared (FTIR) spectra were obtained using a Bomem MB100 infrared spectrometer. UV−vis measurements were carried out using a Shimadzu UV-1601PC UV−vis spectrophotometer.

The synthesis and characterization of Pluronic−PAA copolymer have been described in detail elsewhere.[9,27,29,31,32,34,41,46] The synthesis of dye-labeled Pluronic−PAA was carried out as follows: Pluronic−PAA copolymer was dissolved in deionized water at 4 °C overnight. Following re-equilibration at room temperature, the pH was adjusted to 9.0 by adding 5 M NaOH solution. An aqueous solution containing 80 mg of fluoresceinamine and 400 mg of 1-[(3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, 5 mL total, was added to 45 mL of Pluronic−PAA solution (3.3 wt %, pH 9.0), and the resulting mixture was gently stirred in the dark at 8 °C overnight. The resulting brightly yellow solution was exhaustively dialyzed against deionized water (pH adjusted to 7.4) using a Spectra/Por cellulose ester membrane (molecular weight cutoff, 10 000 Da) until no dye was spectrophotometrically detectable in the dialyzate. The dialyzed, labeled Pluronic−PAA copolymer was lyophilized and stored at −20 °C in the dark.

**Procedures.** Pluronic−PAA solutions were prepared by weighing the appropriate amount of polymer powder and dissolving in purified water. Solutions were stirred overnight to ensure dissolution. The pH was adjusted to 7.00 ± 0.02 by adding the required volume of 10 M NaOH. Solutions were stored at 4 °C for 48 h before use.

Diffusion coefficients were measured by cyclic voltammetry using a standard three-electrode electrochemical cell immersed in a water bath with the temperature controlled to ±0.1 °C. Experiments were typically performed in 1 mL solutions. Cyclic voltammograms were obtained for at least five scan rates (between 5 and 100 mV/s), and peak currents were measured after background subtraction. A plot of the peak current ($i_p$) versus the square root of the scan rate ($v^{1/2}$) was prepared, and the diffusion coefficient was calculated from the slope of this plot, in accordance with the Randles−Sevcik equation,[54]

$$i_p = (2.69 \times 10^5) n^{3/2} A C D^{1/2} \quad (1)$$

where $i_p$ is the peak current, $n$ is the number of electrons transferred in the electrode reaction, $A$ is the electrode area, $D$ is the diffusion coefficient of the redox-active species, $C$ is the concentration of redox-active species, and $v$ is the scan rate. All measurements were carried out at least in triplicate, and error bars (where shown) represent the standard deviation of the measurements. Electrochemistry of the $FcOHC_x$ probes was complicated by adsorption at the electrode surface, and therefore only the oxidative peak current was used to calculate diffusion coefficients for these species. Platinum working electrodes were used for measurements involving ferricyanide, AcFc, and $FcOHC_x$ probes.

For the electrochemical experiments involving cytochrome *c*, gold electrodes were modified with either 4,4′-bipyridyl or 4-mercaptopyridine, using a method based on that of Bond and co-workers,[55,56] in which electrodes were modified by immersion in solutions of the appropriate modifier. Electrodes were immersed for 10 min in a 1 mM solution of 4-mercaptopyridine, followed by drying in air. The electrode response was stable to repeated potential cycling (up to 10 repeats), enabling cyclic voltammograms to be obtained at different scan rates using the same electrode surface. Amphiphilic ferrocene alcohols were synthesized based on the procedure of Zu and Rusling,[57] utilizing a reaction between Grignard reagents, formed from the appropriate alkyl bromide (1-bromooctane, 1-bromododecane, or 1-bromohexadecane), and acetylferrocene. The products were purified by column chromatography (silica gel 60) and characterized by $^1$H NMR and FTIR. NMR data were in agreement with those reported by Zu and Rusling.[57]

Release experiments were carried out under unstirred conditions as follows: A known volume (generally 300 $\mu$L) of Pluronic−PAA hydrogel loaded with the required solute was placed in the bottom of a cuvette of path length 1 cm and equilibrated at 37 °C to allow the hydrogel phase to form. A known volume of buffer solution was also equilibrated at 37 °C and then added to the hydrogel in the cuvette at $t = 0$. Great care was taken to avoid disturbing the surface of the hydrogel. The amount of solute in the aqueous phase was monitored at regular intervals by measuring the absorbance at the appropriate wavelength. Using this method, it was possible to measure the absorbance of the aqueous phase in situ, avoiding the necessity to withdraw and replace aliquots of the aqueous phase. At the end of the experiment, the aqueous and buffer phases were cooled and mixed before measuring the final concentration of solute in the system. In the cytochrome *c* release experiments, a control was included by maintaining a cytochrome *c* sample of known concentration in Pluronic−PAA hydrogel at 37 °C and monitoring the absorbance at 408 nm to ensure that the protein was stable over the time period involved. $\lambda_{max}$ values of the ferrocene-based probes were 462, 443, 446, and 447 nm for AcFc, $FcOHC_{10}$, $FcOHC_{14}$, and $FcOHC_{18}$, respectively, with corresponding extinction coefficients of 587, 106, 115, and 117 $M^{-1}$ $cm^{-1}$, respectively.

To differentiate between the effects of erosion of the hydrogel and diffusion of the probe through the network structure of the hydrogel, similar experiments without probe species were carried out using a fluoresceinamine-labeled form of the Pluronic−PAA (Fl−Pluronic−PAA). This enabled measurement of the rate of erosion of the hydrogel by the various buffer solutions used in the release experiments, using the absorbance at 492 nm to measure the concentration of Fl−Pluronic−PAA in the buffer phase. The rate of release due to diffusion ($R_{diff}$) was then estimated by subtracting the cumulative erosion ($R_{eros}$) at a given time from the corresponding value for total cumulative release ($R_{tot}$), assuming that

$$R_{tot} = R_{eros} + R_{diff} \quad (2)$$

As the erosion data were obtained in the absence of probe species, this approach does not account for the effect of the probe molecules on the hydrogel structure. While it has been shown that hydrophobic solutes can affect the aggregation behavior of self-assembling hydrogels,[58] the small probe concentrations (~2 mM) used here should minimize such effects.

## Results and Discussion

**Diffusion of Water-Soluble Probes.** The diffusion coefficients of ferricyanide ($D_{ferri}$) in 1% solutions of

(54) Bard, A. J.; Faulkner, L. R. *Electrochemical Methods*: *Fundamentals and Applications*; Wiley: New York, 1980; p 218.

(55) Bond, A. M.; Hill, H. A. O.; Komorsky-Lovric, S.; Lovric, M.; McCarthy M.; Psalti, I. S. M.; Walton, N. J. *J. Phys. Chem.* **1992**, *96*, 8100−8105.

(56) Bond, A. M.; Colton, R.; D'Agostino, A.; Traeger, J. C.; Downard, A. J.; Canty, A. J. *Inorg. Chim. Acta* **1998**, *267*, 281−291.

(57) Zu, X.; Rusling, J. F. *Langmuir* **1997**, *13*, 3693−3699.

(58) Nagarajan, R. *Colloids Surf., B* **1999**, *16*, 55−72.

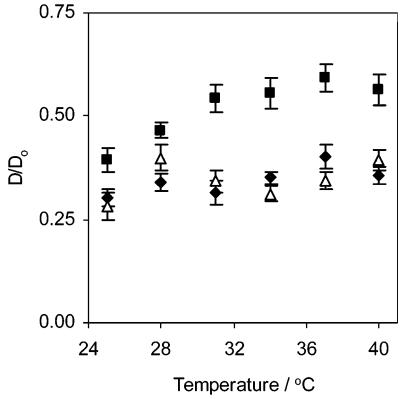


**Figure 1.** Diffusion coefficient ratio of ferricyanide in 1% aqueous solutions of Pluronic−PAA (■), PAA (△), and PEO (◆) as a function of temperature. Values are given as the ratio of the measured diffusion coefficient ($D$) to the corresponding value measured in aqueous solution at the same temperature ($D_0$).

Pluronic−PAA, PEO, and PAA were determined by cyclic voltammetry. Data were obtained over the range 25−40 °C (Figure 1), a temperature range spanning the region in which Pluronic−PAA solutions transform from free-flowing liquids to viscoelastic gels. For the solutions of component polymers (PEO and PAA), little change in the value of $D_{\text{ferri}}$ over this temperature range was observed. In each case, $D_{\text{ferri}}$ increased slightly with increasing temperature. These results are in agreement with the Stokes−Einstein equation for diffusion in an ideal solution,

$$D = kT/6\pi\eta R_{\text{h}} \qquad (3)$$

where $k$ is the Boltzmann constant, $T$ is the absolute temperature, $\eta$ is the local solution viscosity, and $R_{\text{h}}$ is the hydrodynamic radius of the diffusing species. The $D_{\text{ferri}}$ decreased slightly compared to its value in aqueous solution ($D_{\text{ferri,25 °C}} = 7.84 \times 10^6$ cm$^2$/s[59]), presumably due to the increased viscosity of the polymer solutions. The $D_{\text{ferri}}$ also increased across the temperature range in 1% Pluronic−PAA, despite the dramatic increase in viscosity associated with the transition to the hydrogel phase. This appears counterintuitive; however, it has been pointed out[46,60,61] that diffusion of low molecular weight solutes in polymeric solutions and gels is dependent on the local microviscosity experienced by the diffusing molecule rather than the solution bulk viscosity. If the diffusing species is located in the aqueous regions of the network, the microviscosity of which decreases with temperature, we would expect the diffusion coefficient to increase with temperature as observed here. Ferricyanide ion, with its high aqueous solubility and charge of −3, is not expected to interact with the hydrophobic micellar cores of Pluronic−PAA hydrogels. Due to its small size (MW ∼ 212 Da), it appears that ferricyanide ion is able to penetrate the intermicelle network of PAA segments without significant retardation.

Contrasting behavior was found in the case of a larger, predominantly hydrophilic molecule, cytochrome *c*. Figure 2 shows the response obtained at modified electrodes in cytochrome *c* solutions at 25 and 40 °C. The anodic and cathodic peak currents decreased substantially, while the peak potentials were unchanged. $D_{\text{cyt }c}$ was measured in phosphate-buffered saline (PBS) and 1% solutions of

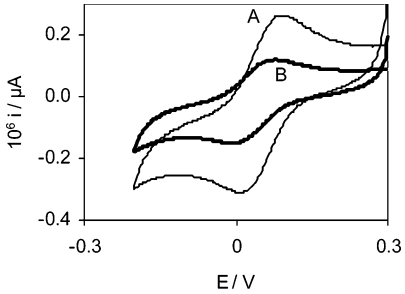


**Figure 2.** Cyclic voltammograms of 0.5 mM cytochrome *c* at a 4-mercaptopyridine-modified gold electrode in 1% Pluronic−PAA solution at 25 °C (A) and 37 °C (B). The scan rate was 50 mV/s.

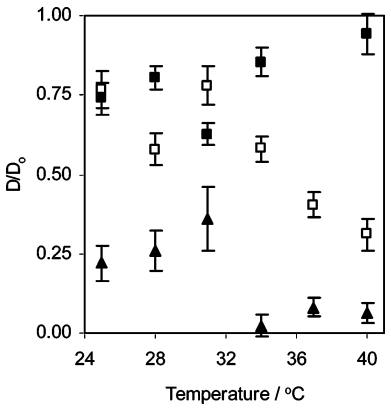


**Figure 3.** Diffusion coefficient ratio of cytochrome *c* in 1% Pluronic−PAA (▲), 1% PEO (■), and 1% PAA (□) as a function of temperature.

Pluronic−PAA, PEO, and PAA (PPO was insoluble in aqueous buffer), and the values obtained are shown in Figure 3 as a function of temperature. There was no change in the electrochemical response obtained on repeated scanning, indicating that there was no significant interaction between the polymers (Pluronic−PAA, PEO, and PAA) and the electrode surface. $D_{\text{cyt }c}$ in PBS was within the range of values reported in aqueous solutions.[62−66] A slight decrease in $D_{\text{cyt }c}$ was observed at higher temperatures in PBS, while $D_{\text{cyt }c}$ increased slightly in 1% PEO. $D_{\text{cyt }c}$ in 1% PAA was lower than in PBS, with a more significant decrease at higher temperatures. At pH 7, approximately 36% of the carboxylic acid groups on the PAA chains are ionized,[34] so that the PAA chains possess a significant negative charge. At this pH, cytochrome *c* has a net positive charge of +9 in the oxidized state and +8 in the reduced state.[67] Thus, electrostatic binding between cytochrome *c* and PAA chains is likely to occur. Such binding, resulting in the joint diffusion of the polymer chains and cytochrome *c*, retards the movement of the protein in solution. Formation of the gel networks, which immobilizes the polymer chains, lowers the diffusion coefficient of the polymer-bound protein still further.

(59) Kakihana, M.; Ikeuchi, H.; Sato, G. P.; Tokudo, K. *J. Electroanal. Chem.* **1980**, *108*, 381−383.
(60) Hyk, W.; Ciszkowska, M. *J. Phys. Chem.* **1999**, *103*, 6466−6474.
(61) Tatischeff, H. B.; Fritsch-Faules, I.; Wrighton, M. S. *J. Phys. Chem.* **1993**, *97*, 2732−2739.
(62) Correia dos Santos, M. M.; Peas de Sousa, P. M.; Simoes Goncalves, M. L.; Lopes, H.; Moura, I.; Moura, J. J. G. *J. Electroanal. Chem.* **1999**, *464*, 76−84.
(63) Ehrenberg, A. *Acta Chem. Scand.* **1957**, *11*, 1257.
(64) Eddowes, M. J.; Hill, H. A. O. *J. Am. Chem. Soc.* **1979**, *101*, 4461−4464.
(65) Eddowes, M. J.; Hill, H. A. O. In *Electrochemical and Spectrochemical Studies of Biological Redox Components*; Kadish, K., Ed.; Advances in Chemistry Series 201; American Chemical Society, Washington, DC, 1982.
(66) Paeschke, M.; Hintsche, R.; Wollenberger, U.; Jin, W.; Scheller, F. *J. Electroanal. Chem.* **1995**, *393*, 131−135.
(67) Stryer, L. *Biochemistry*; Freeman: New York, 1995; p 541.

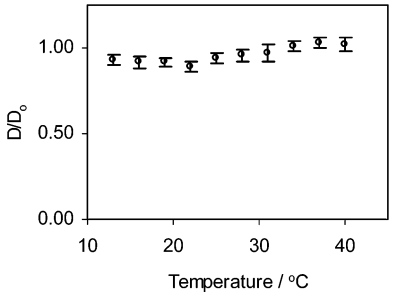


**Figure 4.** Diffusion coefficient ratio of AcFc in 1% Pluronic−PAA hydrogel as a function of temperature.

The decreased value of $D_{\text{cyt } c}$ in 1% Pluronic−PAA solutions compared to PBS or either of the component solutions can be attributed to a combination of increased viscosity of the Pluronic−PAA solution and binding of the protein to the PAA segments. $D_{\text{cyt } c}$ initially increased with temperature but decreased dramatically (factor of ∼15) between 31 and 34 °C. The average value of $D_{\text{cyt } c}$ at temperatures above 31 °C is ca. 5% of that in PBS, indicating that the network structure of Pluronic−PAA in the hydrogel phase represented a significant barrier to the diffusion of cytochrome *c*, a hydrophilic protein with a diameter of ∼3.5 nm. Ho et al.[46] examined the rate of diffusion of the proteins BSA and concanavalin A in Pluronic−PAA solutions and also found that protein diffusion coefficients decreased in the temperature region where the gel began to form. This effect was attributed to hindered diffusion as the proteins negotiated a more torturous diffusional path through the gel structure. Since cytochrome *c* is a hydrophilic protein and hence unlikely to be absorbed into the micellar aggregates, the observed decrease in $D_{\text{cyt } c}$ can be attributed to obstruction by the network of the Pluronic−PAA hydrogels.

**Diffusion of Ferrocene-Based Probes.** The diffusion coefficients of ferrocene derivatives (AcFc, $FcOHC_{10}$, $FcOHC_{14}$, and $FcOHC_{18}$) were determined over the range 13−40 °C in order to examine possible effects of small nonmicellar aggregates which exist in Pluronic−PAA solutions at temperatures below the cmt.[31] These aggregates form at temperatures above 18 °C[31] due to hydrophobic interactions between PPO segments. The diffusion profile of AcFc in 1% Pluronic−PAA hydrogels is shown in Figure 4, with $D_{\text{AcFc}}$ being essentially constant over the range 13−25 °C and increasing slightly above this temperature range. Since the ferrocene structure is inherently hydrophobic, with low aqueous solubility, AcFc would be expected to be solubilized in the hydrophobic cores of the micellar aggregates, which would presumably result in a decrease in the measured value of $D_{\text{AcFc}}$. The results suggest that this is not the case, that is, that AcFc does not seem to be located in the micelle core. Since $D_{\text{AcFc}}$ does not undergo a decrease when gelation occurs in Pluronic−PAA solutions, it appears that AcFc is either predominantly located in the aqueous bulk or more likely loosely associated with the PEO-containing inner corona of the micelle structures. AcFc has a MW of 228 Da, and in the absence of strong hydrophobic interactions with the micellar regions, diffusion through the gel network is likely to be essentially unhindered, as in the case of ferricyanide.

The structure of the synthesized ferrocene derivatives with alcohol-bearing alkyl side chains is shown in Figure 5, using $FcOHC_{14}$ as an example. The voltammetric responses of $FcOHC_{10}$ and $FcOHC_{18}$ at 13 and 37 °C are shown in Figure 6. The diffusion profiles of these $FcOHC_x$ probes in Pluronic−PAA solutions (Figure 7) differ

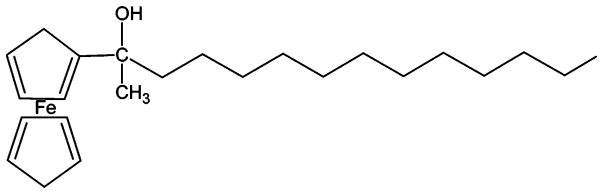


**Figure 5.** Structure of $FcOHC_{14}$.

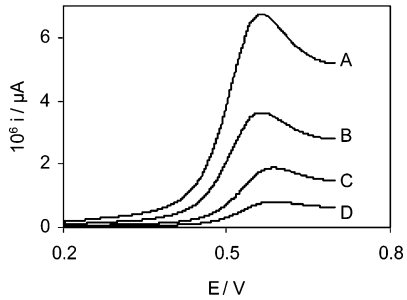


**Figure 6.** Voltammetric response of $FcOHC_{10}$ in 2% Pluronic−PAA hydrogel at 13 °C (A) and 37 °C (B) and of $FcOHC_{18}$ at 13 °C (C) and 37 °C (D).

significantly from that of AcFc. Table 1 lists the values of the $FcOHC_x$ diffusion coefficients at 13 and 37 °C in PBS (with 2% Tween surfactant to enhance solubility) and 1% and 2% hydrogels and gives the dodecane−water distribution coefficients, as reported by Zu and Rusling.[57]

The values obtained for $D_{\text{FcOHC}_x}$ in PBS−Tween and 1% Pluronic−PAA are similar, indicating that the probes freely diffuse in the latter solution. Similar trends in the diffusion coefficient temperature profiles were observed for each of the $FcOHC_x$ probes. In PBS−Tween, $D_{\text{FcOHC}_x}$ increased slightly between 13 and 37 °C for each probe. In Pluronic−PAA solutions, $D_{\text{FcOHC}_x}$ decreased dramatically over the temperature range 16−25 °C, before leveling off at temperatures above 28 °C (Figure 7a−c). The extent of the reduction in $D_{\text{FcOHC}_x}$ increased with increasing alkyl chain length of the probe and also with increasing Pluronic−PAA concentration. For example, the ratios of the diffusion coefficients at 13 and 37 °C ($D_{13}/D_{37}$, Table 1) were 3.4, 25.9, and 50.8 in 2% Pluronic−PAA for $FcOHC_{10}$, $FcOHC_{14}$, and $FcOHC_{18}$, respectively. For $FcOHC_{14}$, $D_{13}/D_{37}$ was 0.8 in the PBS−Tween solution and 14.3 in 1% Pluronic−PAA solution.

The decrease in $D_{\text{FcOHC}_x}$ coincides with a dramatic increase in the viscosity of the Pluronic−PAA system as shown in Figure 8. Since the size of the various $FcOHC_x$ probes did not vary dramatically, the decrease in $D_{\text{FcOHC}_x}$ at elevated temperature can be attributed to solubilization of the probes into the hydrophobic cores of the micellar aggregates, which are formed in Pluronic−PAA solutions at elevated temperatures. As shown by the dodecane−water distribution coefficients in Table 1, the hydrophobicity of these materials increases significantly with increasing alkyl chain length. Kozlov et al.[68] examined the partitioning of a series of alkyl derivatives of fluorescein in Pluronic copolymer solutions and showed that the fluorescence intensity decreased with increasing alkyl chain length due to incorporation of the more hydrophobic probes into the micellar cores. Melik-Nubarov and Kozlov[69] characterized the micelles of Pluronic copolymers using hydrophilic (doxorubicin) and hydrophobic (perylene) fluorescent probes and showed that the hydrophobic probe

(68) Kozlov, M. Y.; Melik-Nubarov, N. S.; Batrakova, E.; Kabanov, A. V. *Macromolecules* **2000**, *33*, 3305−3313.
(69) Melik-Nubarov, N. S.; Kozlov, M. Y. *Colloid Polym. Sci.* **1998**, *276*, 381−387.

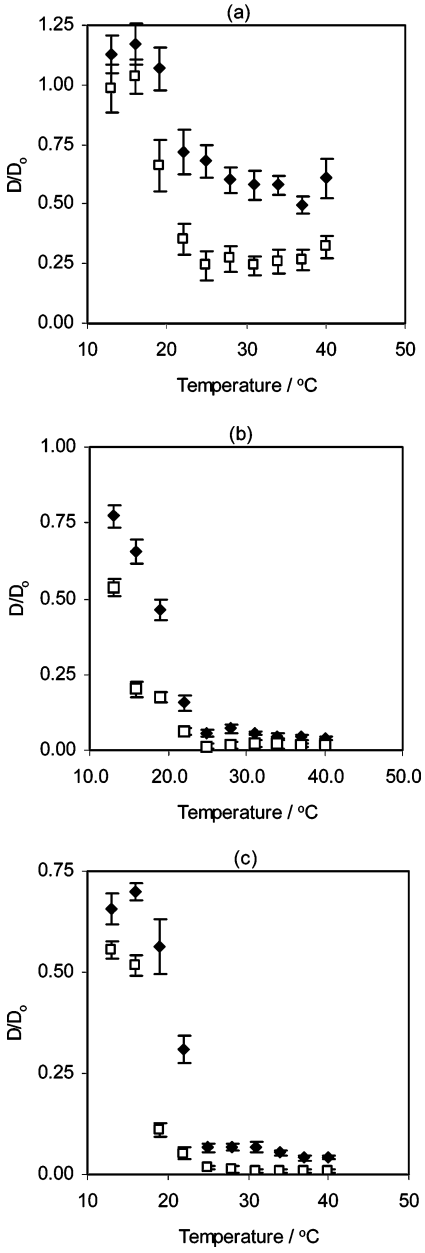


**Figure 7.** Diffusion coefficient ratios of $FcOHC_{10}$ (a), $FcOHC_{14}$ (b), and $FcOHC_{18}$ (c) in 1% Pluronic−PAA (◆) and 2% Pluronic−PAA (□) as a function of temperature.

was solubilized to a much greater extent than the hydrophilic one. These reports are in agreement with the results described here. The behavior of the $FcOHC_x$ probes in Pluronic−PAA solutions can be contrasted with that of AcFc (Figure 4) which did not exhibit a decrease in diffusion coefficient at elevated temperatures and therefore was presumably not solubilized in the micellar aggregates. The $FcOHC_x$ behavior in Pluronic−PAA solutions is also in contrast to their behavior in the nongelling PBS−Tween solutions, in which a slight increase with temperature was observed (Table 1).

While the general trend of increased solubilization into hydrophobic micellar cores as alkyl chain length increased agrees with intuitive expectations, some details of the behavior observed are more difficult to rationalize. The reduction in diffusion coefficient was relatively modest for $FcOHC_{10}$ but much more significant for $FcOHC_{14}$ and $FcOHC_{18}$, even though the dodecane−water distribution coefficients indicated (Table 1) that the greatest difference in hydrophobicity was between $FcOHC_{14}$ and $FcOHC_{18}$. The $FcOHC_x$ probes have three components: the aromatic and hydrophobic ferrocene moiety, the alkyl side chain, and the polar −OH group. Nagarajan et al.[70,71] explored the solubilization of hydrophobic solutes in PEO−PPO and PEO−PPO−PEO copolymers. They found that much higher amounts of aromatic hydrocarbons were solubilized compared to aliphatic hydrocarbons. Moreover, solubilization of linear alkanes decreased with increasing chain length. When one also considers the effect of the polar −OH group, which renders the $FcOHC_x$ molecules somewhat amphiphilic, it is apparent that a complex balance of effects may be in action. Given the amphiphilic nature of $FcOHC_x$ and the moderate effects on diffusion coefficients (compared to $D$ values of $\sim 10^{-14}$ cm$^2$/s reported for hydrophobic fluorescent probes incorporated in micellar cores[41]), it is likely that the $FcOHC_x$ molecules were solubilized at the core−corona interface of the micellar aggregates rather than in the core interior. Since the cores of the micelles in the network are immobile, diffusion of the species solubilized into micelles will be reduced. However, some fraction of the $FeOHC_{18}$ is not solubilized but only entrapped in the network and thus has greater freedom to diffuse more rapidly. Observed decreases in the diffusion coefficients of probe molecules could be due to (a) a uniform decrease in the actual diffusion coefficient of the probe molecule due to obstruction by the hydrogel network, (b) binding of a certain fraction of the probe molecules to the hydrogel network, which then exhibit reduced (or zero) diffusion coefficients while the remaining unbound probe molecules diffuse relatively freely, or (c) a combination of the two mechanisms. The techniques used here do not allow us to distinguish between the various mechanisms of diffusion described above. However, since obstruction of small molecules (ferricyanide ion and acetylferrocene) was not observed, mechanism b would appear to be the most likely mechanism in operation in this case.

**Release of Cytochrome *c*.** The release of cytochrome *c* from 1% and 2% Pluronic−PAA hydrogels into aqueous solutions was monitored by UV−vis spectroscopy under unstirred conditions. Cytochrome *c* release was examined using a range of 0.025−1 M NaCl in the buffer phase (Figure 9a,b). Salt concentration had a significant effect on the rate of release of cytochrome *c* from the hydrogel. At low salt concentrations, an initially fast rate of release gave way to an approximately constant rate of release from the gel. Under the unstirred conditions used here, approximately 50% of the total amount of protein was released after 48 h. As the salt concentration was increased, the initial release rate of the protein increased dramatically, with total release from the 2% hydrogel after 1 h increasing from ∼8% for 25 mM NaCl to ∼43% for 1 M NaCl. In the latter case, the release was essentially complete after 12−15 h. Bromberg et al.[34] have shown that increasing the salt concentration forces PPO segments to aggregate at lower temperatures. This is also a well-reported feature of hydrogels formed in solutions of Pluronic copolymers,[72−75] which also form viscoelastic gels

(70) Nagarajan, R.; Barry, M.; Ruckenstein, E. *Langmuir* **1986**, *2*, 210−215.
(71) Nagarajan, R.; Ganesh, K. *J. Colloid Interface Sci.* **1996**, *184*, 489−499.
(72) Pandit, N. K.; Wang, D. *Int. J. Pharm.* **1998**, *167*, 183−189.
(73) Jain, N. J.; George, A.; Bahadur, P. *Colloids Surf., A* **1999**, *157*, 275−283.
(74) Jain, N. J.; Aswal, V. K.; Goyal, P. S.; Bahadur, P. *Colloids Surf., A* **2000**, *173*, 85−94.
(75) Desai, P. R.; Jain, N. J.; Sharma, R. K.; Bahadur, P. *Colloids Surf., A* **2001**, *178*, 57−69.

**Table 1. $FcOHC_x$ MWs and Diffusion Coefficients in 1% and 2% Pluronic−PAA Solutions at 13 and 37 °C**

| probe | MW (Da) | $P^a$ | solution | $D$ ($cm^2/s$) 13 °C | $D$ ($cm^2/s$) 37 °C | $D_{13}/D_{37}{}^b$ |
|---|---|---|---|---|---|---|
| $FcOHC_{10}$ | 346.32 | 180 | PBS + 2% Tween | $7.9 \times 10^{-6}$ | $8.5 \times 10^{-6}$ | 0.9 |
| | | | 1% Pluronic−PAA | $8.9 \times 10^{-6}$ | $4.2 \times 10^{-6}$ | 2.1 |
| | | | 2% Pluronic−PAA | $7.8 \times 10^{-6}$ | $2.3 \times 10^{-6}$ | 3.4 |
| $FcOHC_{14}$ | 398.39 | 270 | PBS + 2% Tween | $1.4 \times 10^{-6}$ | $1.8 \times 10^{-6}$ | 0.8 |
| | | | 1% Pluronic−PAA | $1.1 \times 10^{-6}$ | $7.7 \times 10^{-8}$ | 14.3 |
| | | | 2% Pluronic−PAA | $7.5 \times 10^{-7}$ | $2.9 \times 10^{-8}$ | 25.9 |
| $FcOHC_{18}$ | 450.46 | 1250 | PBS + 2% Tween | $1.1 \times 10^{-6}$ | $1.3 \times 10^{-6}$ | 0.8 |
| | | | 1% Pluronic−PAA | $7.2 \times 10^{-7}$ | $5.3 \times 10^{-8}$ | 13.6 |
| | | | 2% Pluronic−PAA | $6.1 \times 10^{-7}$ | $1.2 \times 10^{-8}$ | 50.8 |

[a] *P* values are the dodecane/water distribution coefficients reported by Zu and Rusling (ref 57). [b] $D_{13}/D_{37}$ represents the ratio of the diffusion coefficient for each probe at 13 and 37 °C.

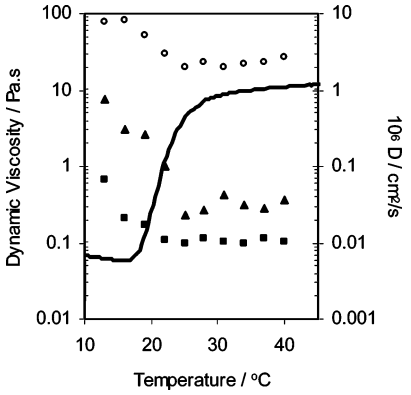


**Figure 8.** Diffusion coefficients of $FcOHC_{10}$ (○), $FcOHC_{14}$ (▲), and $FcOHC_{18}$ (■) in 2% Pluronic−PAA solution as a function of temperature. The solid line is the dynamic viscosity of the solution as a function of temperature.

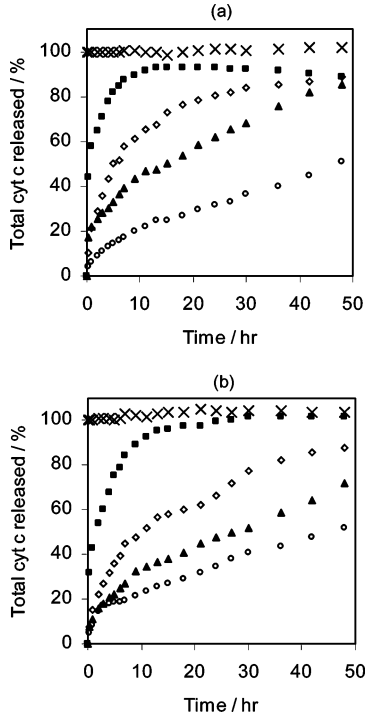


**Figure 9.** Plot of the amount of cytochrome *c* released (%) from (a) 1% Pluronic−PAA hydrogel and (b) 2% Pluronic−PAA hydrogel as a function of time for 25 mM (○), 100 mM (▲), 200 mM (◇), and 1 M (■) NaCl. A control sample (×) showing the stability of the protein over the same time scale is also shown.

due to formation of micellar aggregates at elevated temperatures, albeit at significantly higher concentrations than those for Pluronic−PAA. Ho et al.[46] examined diffusion of the protein BSA in Pluronic−PAA hydrogels. A decrease in diffusion coefficient was observed coinciding with gelation of the Pluronic−PAA. The decrease in protein diffusion rate was attributed not to partition into hydrophobic micellar cores but rather to hindered diffusion effects as the proteins negotiated a more torturous path through the structured gel. Cytochrome *c* is more hydrophilic in overall terms than BSA, as determined using the grand average of hydropathicity (GRAVY) index.[76] The GRAVY index values for cytochrome *c* and BSA were determined using the ProtParam program[77] as −0.902 and −0.433, respectively, where increasing negative values indicate greater hydrophilicity. Moreover, the structure of cytochrome *c* has been described as a hydrophobic core surrounded by more hydrophilic residues.[78] It is therefore reasonable to suggest that decreased diffusion coefficients of cytochrome *c* in Pluronic−PAA hydrogels are also due to obstruction by the network structure rather than by absorption into hydrophobic micellar cores.

As shown by the electrochemical measurements of $D_{\text{cyt } c}$ discussed above, electrostatic interactions between the positively charged protein and the negatively charged carboxylic acid groups of PAA segments are likely to represent a significant hindrance to diffusion of cytochrome *c* in Pluronic−PAA solutions and gels. Increasing the salt concentration provides $Na^+$ and $Cl^-$ ions, which are capable of screening the electrostatic interaction between cytochrome *c* and PAA, rendering the cytochrome *c* molecules increasingly free to diffuse through the hydrogel network, as only the obstruction effect of the polymer network restricts diffusion of the protein.

The release of an uncharged species (AcFc) from a 1% Pluronic−PAA solution was also examined over the same range of NaCl concentrations (Figure 10). No significant effect on the rate of release is observed in this case. The absence of a salt effect on the release rate of uncharged species is compatible with the release mechanism for cytochrome *c* suggested above. Charge effects have been shown to have a profound influence on release of charged species from hydrogels. Lee and Chiu[79] examined charge effects for a range of solutes in neutral, anionic, and cationic poly(*N*-isopropylacrylamide) (pNIPAAm) cross-linked gels. The rate of release of a neutral solute (caffeine) was not significantly affected by the ionic nature of the hydrogel but was determined by the swelling ratio of the gels. In contrast, the release of charged solutes such as crystal violet (cationic) and phenol red (anionic) was dramatically influenced by the charge on the hydrogel.

(76) Kyte, J.; Doolittle, R. F. *J. Mol. Biol.* **1982**, *157*, 105−132.
(77) The program is available online at http://www.expasy.ch/tools/protparam.html.
(78) Moore, G. R.; Pettigrew, G. W. *Cytochrome c*: *Evolutionary, Structural and Physiochemical Aspects*; Springer-Verlag: Berlin, 1990.
(79) Lee, W.-F.; Chiu, R.-J. *Mater. Sci. Eng.* **2002**, *20*, 161−166.

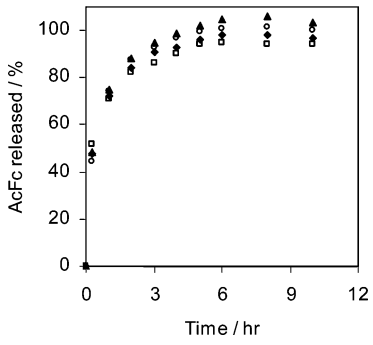


**Figure 10.** Plot of the amount of acetylferrocene releases from 1% Pluronic−PAA hydrogel as a function of time for 25 mM (○), 100 mM (▲), 200 mM (◆), and 1 M (□) NaCl.

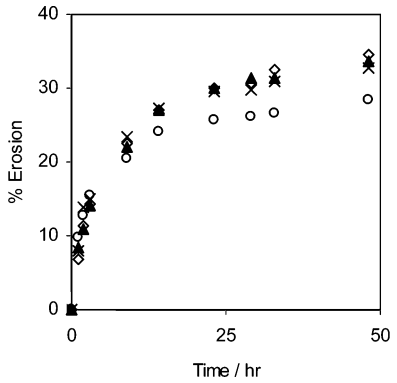


**Figure 11.** Rate of erosion of 2% Fl−Pluronic−PAA hydrogel over time as a function of the NaCl concentration in the buffer phase. The NaCl concentrations used were 25 mM (◇), 100 mM (▲), 200 mM (×), and 1 M (○).

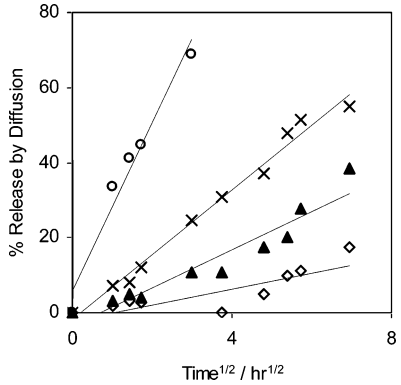


**Figure 12.** Rate of diffusional release of cytochrome (%) from 2% Pluronic−PAA hydrogel as a function of time$^{1/2}$ for 25 mM (◇), 100 mM (▲), 200 mM (×), and 1 M (○) NaCl.

Figure 11 shows the erosion of a 2% Pluronic−PAA hydrogel over time as a function of the NaCl concentration in the buffer phase. There was a slight decrease in the rate of erosion as the ionic strength of the buffer phase was increased, most notably for the 1000 mM NaCl solution. This effect may be due to the increased aggregation of the hydrophobic PPO segments at higher salt concentrations, as described by Bromberg et al.[27] By subtracting the data in Figure 11 from the corresponding total cumulative release data (Figure 9b), we obtained the cumulative release by diffusion. These data are shown in Figure 12 as a function of time$^{1/2}$. This plot can be used to test whether the system adheres to the Higuchi square root law,[80] derived for the diffusional release of solute from one side of a semisolid slab, which is given by

$$Q = 2C_0(Dt/\pi)^{1/2} \quad (4)$$

where $Q$ is the amount of solute released per unit area, $C_0$ is the initial solute concentration, $D$ is the solute diffusion coefficient, and $t$ is time. If solute release obeys this law, the amount of solute released is a linear function of $t^{1/2}$.

Data for buffers with high ionic strengths (200 and 1000 mM) show good fit to the linear model, with $R^2$ values of 0.9867 and 0.9632, respectively. The amounts of cytochrome *c* released by diffusion through the hydrogel were determined to be 55% and 73% for 200 and 1000 mM NaCl concentrations, respectively, suggesting that the diffusional mechanism is important in these systems. The data for release of cytochrome *c* into buffer with 25 mM NaCl show relatively poor fits to the linear model ($R^2 = 0.6559$). This indicates that diffusion is not the major mechanism controlling the release of cytochrome *c* molecules in this case. After 48 h, the extent of erosion of the hydrogel by this buffer solution was approximately 35% (Figure 11). The total release was 52% (Figure 9b); hence the diffusional release was only 17% of the total amount of cytochrome *c* present. The 100 mM buffer selection gave intermediate results, with an $R^2$ value of 0.9158 and 39% diffusional release. These results are compatible with the mechanism suggested above, wherein release of cytochrome *c* was influenced by shielding of electrostatic interactions between the protein and the polymer network. Figure 12 shows clearly that diffusion played an increasingly important role in the release of cytochrome *c* as the ionic strength was increased, even though the erosion of the hydrogel was not greatly affected (Figure 11).

The diffusion coefficients of cytochrome *c* (Table 2) were calculated from the slopes of the linear portions of the diffusional release versus $t^{1/2}$ plots (shown for 2% Pluronic−PAA hydrogel in Figure 12), based on the equation

$$M/M_\infty = 4/L(Dt/\pi)^{1/2} \quad (5)$$

where $M/M_\infty$ is the fractional release of solute at time $t$ and $L$ is the slab thickness. It has been reported that $D$ can be obtained with ∼5% precision by this method.[81] A number of observations can be made based on the data in Table 2. As already observed, the effect of salt concentration was dominant over that of Pluronic−PAA concentration, as $D_{\mathrm{cyt}\ c}$ values in 1% and 2% hydrogels were approximately equal for each NaCl concentration. $D_{\mathrm{cyt}\ c}$ at low salt concentrations was similar to the values obtained at elevated temperatures ($T$ > 31 °C) by cyclic voltammetry (Figure 3). At higher salt concentrations, $D_{\mathrm{cyt}\ c}$ obtained from release data was approximately equal to that obtained by cyclic voltammetry at temperatures below the gel point. This supports the suggestion that diffusion of cytochrome *c* in Pluronic−PAA solutions and gels is strongly affected by electrostatic interactions.

**Release of Ferrocene Derivatives.** The release of ferrocene derivatives from 1% and 2% Pluronic−PAA hydrogels into aqueous solution was monitored by UV−vis spectroscopy in the aqueous phase. To ensure that release of the more hydrophobic materials was not hindered due to low aqueous solubility, Tween 60 surfactant was added to the aqueous phase at a concentration of 2 wt %. Figure 13 shows the rate of erosion of a 2% Fl−Pluronic−PAA hydrogel over time as a function of the concentration of Tween surfactant in the buffer phase. The rates of erosion were greater than observed for the

(80) Higuchi, W. I. *J. Pharm. Sci.* **1962**, *51*, 802−804.

(81) Favre, E.; Girard, S. *Eur. Polym. J.* **2001**, *37*, 1527−1532.

**Table 2. Diffusion Coefficients of Cytochrome *c* Obtained from Release Experiments**

| Pluronic−PAA concn (% w/w) | NaCl concn (mM) | $D_{cyt\ c}$ (cm²/s) |
|---|---|---|
| 1 | 25 | $2.3 \times 10^{-8}$ |
| | 100 | $6.1 \times 10^{-8}$ |
| | 200 | $2.2 \times 10^{-7}$ |
| | 1000 | $2.1 \times 10^{-7}$ |
| 2 | 25 | $2.4 \times 10^{-8}$ |
| | 100 | $4.6 \times 10^{-8}$ |
| | 200 | $8.1 \times 10^{-8}$ |
| | 1000 | $2.3 \times 10^{-7}$ |

**Table 3. Time Taken for the Plateau Level of Release To Be Reached for Ferrocene-Based Probes**

| probe | Pluronic−PAA concn (% w/w) | $t_{plateau}$ (h) | probe | Pluronic−PAA concn (% w/w) | $t_{plateau}$ (h) |
|---|---|---|---|---|---|
| AcFc | 1 | 8 | $FcOHC_{14}$ | 1 | 8 |
| | 2 | 10 | | 2 | 24 |
| $FcOHC_{10}$ | 1 | 6 | $FcOHC_{18}$ | 1 | 8 |
| | 2 | 18 | | 2 | 32 |

more polar buffer phases used earlier (Figure 11) and increased with increasing Tween concentration, suggesting that the solubility of Pluronic−PAA copolymer in the buffer phase is increased by the presence of Tween surfactant.

The rate of release of AcFc followed a similar profile as observed for cytochrome *c* at high salt concentration and was essentially complete after ~8 h, at which time approximately 25% of the hydrogel was eroded (Figure 13). The concentration of Pluronic−PAA did not significantly affect the rate of release. These results are in agreement with the diffusion coefficient data discussed above, which suggested that AcFc was not absorbed into micelle interiors in the hydrogel but was instead released by diffusion through the aqueous portions of the hydrogel.

A different release mechanism applies for the $FcOHC_x$ probes (Figure 14a,b). After an initial burst release of 14−28% of the total amount within the first 30 min, there was a significant region in which the rate of release was approximately constant, followed by a plateau region as the amount released neared completion. The rate of release in the "constant rate" region decreased with increasing alkyl chain length of the probe. Since the probe hydrophobicity increases strongly with chain length, it is reasonable to suggest that this reflects increasing solubilization of longer chain $FcOHC_x$ molecules into the micellar regions of the hydrogel. This suggestion is supported by the electrochemical measurements of $D_{FcOHC_x}$, which showed that probe diffusion coefficients decreased with increasing hydrophobicity. The release of $FcOHC_x$ species was strongly affected by Pluronic−PAA concentration, which also supports this argument, as increasing the polymer concentration increases the concentration of micelles which can then absorb greater amounts of the probes. The rate of erosion of the 2% hydrogel is also shown in Figure 14b. The overall release was greater than the rate of erosion in each case, showing that there is significant diffusion of each of the $FcOHC_x$ species through the hydrogel. Table 2 summarizes the approximate times at which each probe reaches its plateau level in 1% and 2% hydrogels and shows that the 2% hydrogels have the ability to significantly retard the release of probe molecules. The time required for maximum release increased as the alkyl chain length and hydrophobicity of the probe increased. The diffusional component of $FcOHC_x$ release was obtained by subtracting the release due to erosion from the overall release (as described in the Experimental Section). Release of $FcOHC_x$ by

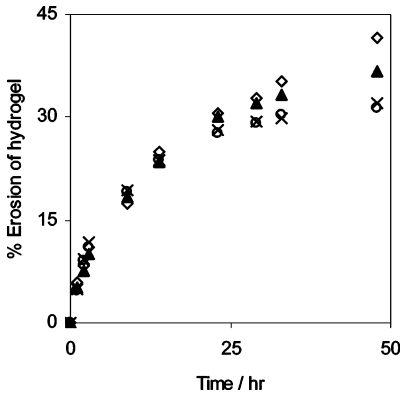


**Figure 13.** Rate of erosion (5) of a 2% Fl−Pluronic−PAA hydrogel over time as a function of the concentration of Tween surfactant in the buffer phase. The Tween concentrations used were 0.5% (○), 1% (×), 2% (▲), and 4% (◇).

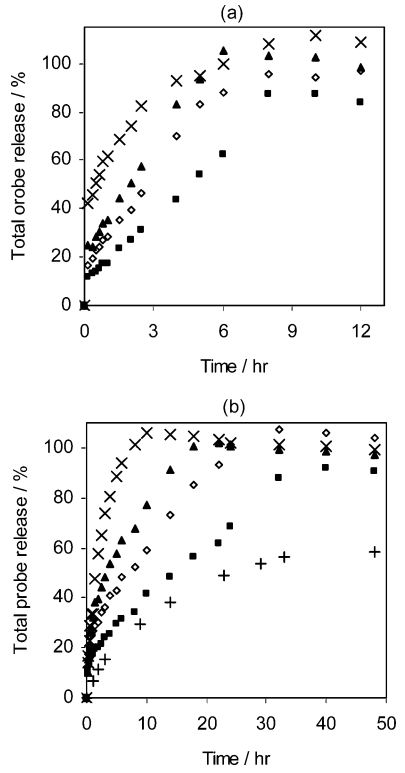


**Figure 14.** Plot of the amount of ferrocene-based probes released from (a) 1% Pluronic−PAA hydrogel and (b) 2% Pluronic−PAA hydrogel. The probes used were AcFc (×), $FcOHC_{10}$ (▲), $FcOHC_{14}$ (○), and $FcOHC_{18}$ (■). The hydrogel erosion (+) is also shown for the 2% hydrogel.

diffusion is shown in Figure 15. The contribution of diffusion decreases with increasing hydrophobicity of the probes, indicating that the more hydrophobic probes are more strongly associated with the micellar regions of the hydrogel and thus more dependent on erosion of the hydrogel structure as a release mechanism, whereas the more hydrophilic probes have greater freedom to diffuse through the hydrogel network.

After data for the initial burst release were removed from the (total) release profiles of the AcFc and $FcOHC_x$ probes, straight lines were observed (shown in Figure 16 for release from 2% hydrogel); that is, over a certain temperature range, release rate was essentially independent of time. These observations are in agreement with the previously reported[28,41] bimodal release kinetics and indicate that Pluronic−PAA hydrogels were capable of

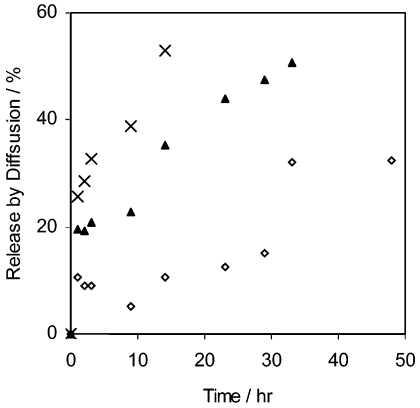


**Figure 15.** Plot of the diffusional component of the rate of release of $FcOHC_{10}$ (×), $FcOHC_{14}$ (▲), and $FcOHC_{18}$ (◇) probes from 2% Pluronic−PAA hydrogel.

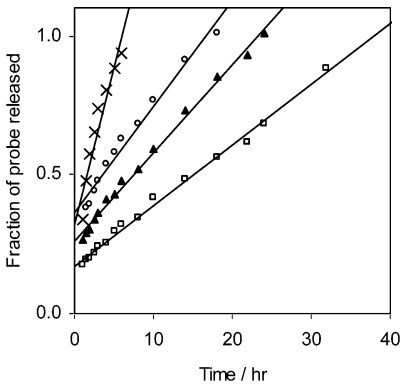


**Figure 16.** Fractional rate of release of AcFc (×), $FcOHC_{10}$ (○), $FcOHC_{14}$ (▲), and $FcOHC_{18}$ (□) vs time (2% Pluronic−PAA gel) for the regions in which approximately constant release rates were observed.

delivering hydrophobic materials at a constant rate over a certain portion of the release profile. In fact, the correlation coefficients obtained show that the linear model was more appropriate for the more hydrophobic probes. AcFc, which was not observed to be associated with the micellar aggregates of Pluronic−PAA hydrogels, gives relatively low $R^2$ (0.9200); $FcOHC_{10}$, the least hydrophobic of the $FcOHC_x$ probes, also showed some deviation from the linear fit ($R^2 = 0.9795$). Electrochemically measured diffusion coefficients for $FcOHC_{10}$ suggested that this probe was only moderately associated with the micellar aggregates. $FcOHC_{14}$ and $FcOHC_{18}$, both of which appear to be more strongly associated with the micellar aggregates, gave excellent fits to the linear model, with $R^2$ values of 0.9934 and 0.9945, respectively.

A further experiment was carried out using AcFc and $FcOHC_{18}$ to verify that low solubility in the aqueous phase was not affecting the release of the ferrocene derivatives. The results of this experiment are shown in panels a and b of Figure 17, respectively. Release profiles similar to those described above were obtained for AcFc and $FcOHC_{18}$, and the rate of release and final amount released were largely unaffected by the concentration of Tween over the range 0.5−4.0 wt %.

## Conclusions

The diffusion of the electrochemically active solutes ferricyanide, cytochrome *c*, and a range of ferrocene derivatives was examined as a function of temperature in solutions and hydrogels of Pluronic−PAA copolymer. The rate of release of the probe molecules from Pluronic−PAA

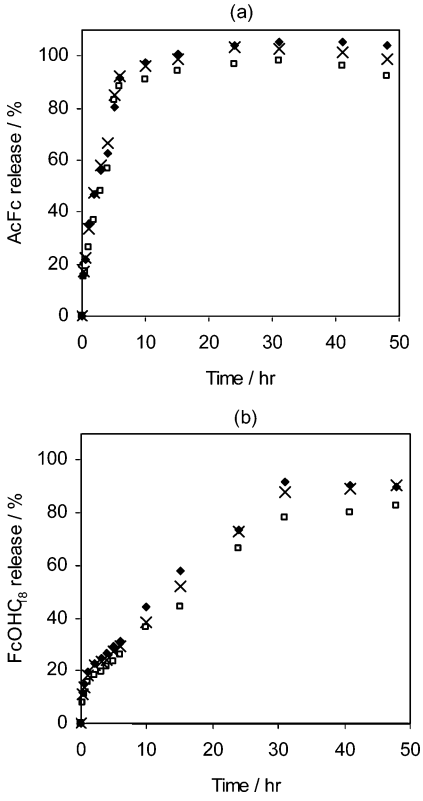


**Figure 17.** Rate of release (%) of AcFc (a) and $FcOHC_{18}$ (b) from 1% Pluronic−PAA hydrogel into aqueous phases containing 0.5% (□), 2% (×), and 4% (◆) Tween surfactant.

hydrogels into aqueous solutions was also studied in an unstirred regime using UV−vis spectroscopy to monitor the release. The contributions of hydrogel erosion and probe diffusion were separated by carrying out erosion experiments using a fluoresceinamine-labeled form of Pluronic−PAA under identical conditions.

The size and hydrophilic/hydrophobic nature of the solutes determined the diffusion and release profiles. The diffusion of ferricyanide was unaffected by the onset of gelation. Despite the large increase in the bulk viscosity of the gel, the small and hydrophilic ferricyanide ion can freely penetrate the hydrogel network. The more hydrophobic probe acetylferrocene (AcFc) might have been expected to be located in the micellar regions of the Pluronic−PAA network and thereby exhibit a decreased rate of diffusion. This was not the case, however, and the rapid release of AcFc from both 1% and 2% hydrogels into aqueous solutions suggested that the AcFc molecules are, at most, loosely associated with the micellar aggregates of the hydrogel.

The more hydrophobic ferrocene derivatives ($FcOHC_x$) are more closely associated with the micelle structures and had lower diffusion coefficients in Pluronic−PAA at elevated temperatures. Their diffusion coefficients were not reduced to the same extent as previously observed for the highly hydrophobic fluorescence probes pyrene and estradiol, which had $D$ values of the order of $10^{-15}$ $cm^2/s$.[21] Due to their amphiphilic nature, the $FcOHC_x$ probes may be located at the core−corona interface rather than fully solubilized in the interior of the core. Following an initial burst effect, the rate of release of these materials from 2% Pluronic−PAA hydrogels into aqueous media was constant over a significant time period. The relative importance of release by hydrogel erosion versus probe diffusion increased with increasing hydrophobicity of the probe species, a further indication that the more hydrophobic

probes were more strongly associated with the micellar hydrogel structure.

At elevated temperatures, the diffusion coefficient of cytochrome *c* decreased dramatically (by a factor of ~15) as gelation occurred in the Pluronic−PAA system. This correlates well with data previously obtained for BSA and concanavalin A and can be attributed to obstruction of diffusing cytochrome *c* molecules by the network of PAA segments. The rate of release of cytochrome *c* from 2% Pluronic−PAA hydrogels was strongly affected by the ionic strength. This sensitivity to ionic strength may represent a limitation in terms of certain drug delivery applications but also provides a potential means of tuning the rate of release. The diffusional portion of the cytochrome *c* release was shown to fit the Higuchi square root law increasingly well at higher salt concentrations, supporting the suggestion that electrostatic binding of cytochrome *c* to the PAA chains of the Pluronic−PAA is a significant factor influencing the mobility of the protein within the hydrogel. In contrast to this, the rate of release of an uncharged species, acetylferrocene, was shown to be largely independent of the ionic strength.

**Acknowledgment.** Funding from the Programme for Research in Third Level Institutions (1999−2000) is gratefully acknowledged.

LA034822W