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Kariyawasam P. Pemawansa[a]; Ishrat M. Khan[a]
[a] Department of Chemistry, Clark Atlanta University, Atlanta, GA

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Water Soluble and Water Stable Non-Covalent Adducts of Sodium Poly(α,L-glutamate)/ Poly(ethylene glycol). Method of Preparation and Characterization of Structure

KARIYAWASAM P. PEMAWANSA AND ISHRAT M. KHAN

Department of Chemistry, Clark Atlanta University, Atlanta, GA

Water soluble and water stable non-covalent adducts of sodium poly(α,L-glutamate) (PGNA, Mol.Wt. 17, 32 and 38 K) and poly(ethylene glycol) (PEG, Mol.Wt. 10 K) were prepared by solution blending. The adducts were purified by a phase separation technique. Two-dimensional (2D)-NOESY ^{1}H-NMR spectroscopy studies show that PEG was non-covalently bound to PGNA by hydrophobic interactions between CH_2 groups of PEG and methylene (both β and γ CH_2) groups of PGNA in an aqueous solution. Because of the nature and strength of hydrophobic interactions between PEG and PGNA, adducts with a different degree of water stability may be prepared. Studies indicate that the PGNA 17K/PEG 10 K adducts were significantly more water stable than the PGNA 32K/PEG 10 K adduct. Prolonged water stability (>2 weeks at 25°C) was observed for the β binding (i.e., PEG bound to inner layer of PGNA molecule) compared to γ binding (i.e. PEG in the outer layer of PGNA).

Keywords water soluble polymers, blends, NMR, differential scanning calorimetry (DSC)

Introduction

Conjugates of bioactive molecules and polymers present significant advantages over non-conjugated counterparts and provide many important added benefits, e.g., water solubility, enhanced circulation times in blood stream, EPR effect towards cancer cells, and being non-viral gene delivery vectors (1–11). The conjugates are normally prepared by covalent linking of the bioactive molecule with a synthetic polymer. However, while covalent conjugation is effective and forms stable conjugated systems, covalent conjugation has some disadvantages. In particular, covalent coupling procedures are costly and require the removal of byproducts that may be toxic. Hence, non-covalent conjugation of a bioactive molecule and a synthetic polymer is attractive, provided that the non-covalent conjugated products are stable. Several papers have reported the non-covalent conjugation of synthetic polymers and peptides/bioactive molecules by non-covalent interactions e.g. hydrophobic and ion-dipole interactions (12–15). In this communication, we report the preparation and characterization of water stable non-covalent adducts

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Address correspondence to Ishrat M. Khan, Department of Chemistry, Clark Atlanta University, Atlanta, GA 30314. E-mail: ikhan@cau.edu

prepared using a water soluble peptide [sodium poly(α,L-glutamate) (PGNA, mol wt 17, 32 and 38 K)] and a water soluble polymer [poly(ethylene glycol) (PEG, mol wt 10].

Poly(ethylene glycol) was selected as the water soluble polymer because of its physicochemical and biological properties that include: a) favorable pharmacokinetics and tissue distribution, b) lack of toxicity and immunogenicity, and c) the ability to excrete through human kidneys when molecular weight is less than 6 K (16, 17). Sodium poly(α,L-glutamate was selected as the water soluble peptide because its β-CH_2 and γ-CH_2 sites are effective in forming hydrophobic interactions with other molecules (14).

Experimental

Preparation of Non-Covalent Adducts

Poly(ethylene glycol) (PEG) [MW 10 K] and PGNA [MW 17 K, 32 K and 38 K], supplied by Sigma Chemical Co. were used without further purification. PEG (1.0 g) and PGNA (0.2 g) were dissolved in 15 ml of water and stirred for 24 h at room temperature. Water was evaporated at 40°C in vacuum, and the white solid (crude product) obtained was further dried at 40°C in vacuum over P_2O_5 for 24 h. Crude products were purified by removing the unbound PEG 10 K by a phase separation technique reported earlier (13). The non-covalent adduct was dissolved in D_2O at 600 mg/ml and allowed to stand overnight at room temperature to yield two layers. The top layer that had unbound PEG was discarded. The bottom layer, which contain pure adduct was isolated and dried at 40°C in vacuum over P_2O_5 overnight. Pure non-covalent adducts of PGNA 17 K, 32 K and 38 K/PEG 10 K were obtained as white solids.

Measurements

Solution NMR spectra were acquired on a Bruker ARX 400 spectrometer at room temperature using 5 mg/ml D_2O solutions. TMS was used as the external standard for obtaining ^{1}H spectra, and sodium chloride was used as the standard for obtaining ^{23}Na spectra (18, 19). All ^{23}Na NMR spectra were recorded using a single pulse acquisition at 90° pulse angle and 2.5 second relaxation delay for 32 acquisitions. The 2D-NOESY ^{1}H-NMR spectra of non-covalent adducts and starting materials (PEG and PGNA) were also acquired on a Bruker ARX 400 spectrometer at room temperature using 10 mg/ml and 5 mg/ml D_2O solutions, respectively. All 2D-NOESY ^{1}H-NMR analyses were carried out using a standard NOESY pulse sequence (13, 14, 20, 21) under the following acquisition conditions:

1024 data points
Relaxation delay − 4.5 ms
Mixing time − 20 ms
Number of scans – 8

where, a relatively short mixing time and small number of data points would allow observation of only the strong interactions that are taking place within a very close proximity.

Glass transition temperature (T_g) and melting temperature (T_m) were determined by differential scanning calorimetry (DSC) using a Perkin-Elmer Pyris 1 DSC, equipped with a thermal data station and Pyris 3.72 software. Solid samples were dried at 60°C

in vacuum over P_2O_5 for a week prior to DSC scans. Solid and solution samples (sealed in Al pans) were scanned at a heating rate of 15°C/min under N_2 to 100°C and quenched cooled at a 320°C/min rate to the starting temperature before obtaining the thermograms.

Circular dichroism (CD) spectra were obtained using a JASCO 710 spectrophotometer at 20 nm/min speed and 0.5 nm resolution.

Results and Discussion

Composition of Non-Covalent Adducts

The composition of the non-covalent adducts was determined by comparing peak areas of ^{1}H-NMR spectra of adducts (Figure 1) to that of a standard mixture of PGNA and PEG. The PEG contents of PGNA 17 K, 32 K and 38 K/PEG 10 K non-covalent adducts ranged between 1.7%–4.8% (w/w), 7.1–16% (w/w) and 6.3–16.9% (w/w), respectively. The PEG content in the adducts increases with increasing molecular weight of PGNA. This observation may be explained in terms of the degree of intermolecular interactions. Poly(sodium glutamate) does not have functional groups capable of forming hydrogen bonds with PEG, and in these adducts, PEG and PGNA in water are held by hydrophobic interactions/contacts (vide supra). Usually, they are reversible and undergo "attach–detach" equilibrium, and for stable binding between the two, a large number of attached/contact points at a given moment is required. As the molecular weight of PGNA increases, the larger peptide macromolecule has a larger number of possible hydrophobic sites per macromolecule for interaction with PEG.

NMR Spectroscopy

The ^{23}Na NMR spectra of non-covalent adducts and the pure PGNA 32 K showed a singlet at 0.1 to –0.02 ppm with 13–16 Hz line width at half height, which is characteristic of free (solvated) sodium ions (18, 19). This is indicative of a lack of ion-dipole interactions between sodium ions of PGNA and oxygen of PEG (Na+—O<) in aqueous solution. However, the 2D-NOESY ^{1}H-NMR spectrum of PEG at a sample concentration of 5 mg/ml in D_20 showed a strong correlation between the CH_2 groups suggesting the presence of intra and/or inter-molecular hydrophobic interactions. This correlation was observed even at concentrations down to 0.01 mg PEG/ml D_2O. The 2D-NOESY ^{1}H-NMR spectrum of pure PGNA (32 K) did not show a correlation suggesting the presence of intra- or inter-molecular interactions in aqueous solutions.

The 2D-NOESY ^{1}H-NMR spectra of the PGNA/PEG non-covalent adducts showed a correlation between CH_2 groups of PEG with (β and γ) CH_2 groups of PGNA. The intensities were dependant on the molecular weight of PGNA. The most intense peak for PGNA 32 K/PEG 10 K and PGNA 38 K/PEG 10 K adducts was the correlation between the CH_2 of the PEG and the γ CH_2 of the PGNA (Figure 2), and the β correlation was seen at the next higher magnification. In general, samples in this series had higher observed intensity (I) for the γ correlation (i.e., $I_\gamma > I_\beta$). However, for adducts having very low amounts of PEG, e.g., PGNA 17 K based adducts having 1.7% (w/w) PEG, the most intense peak was the β correlation ($I_\beta > I_\gamma$) (Figure 3), and the γ correlation was seen at the next higher magnification.

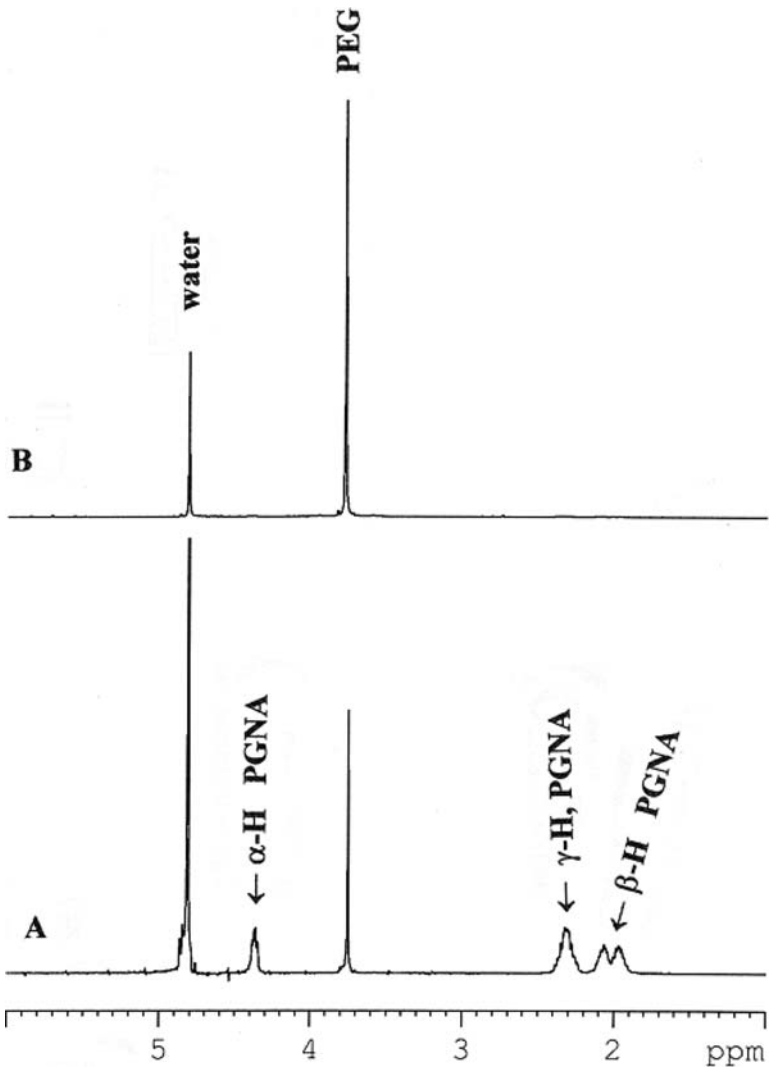

Figure 1. ^{1}H NMR spectra of: A. Bottom layer, B. Top layer obtained after overnight settlement of PGNA 32 K/PEG 10 K crude adduct in D_2O (600 mg/ml) at room temperature.

PGNA Molecular Weight vs. Nature of Binding

As observed by NMR spectroscopy, increasing the molecular weight of the PGNA results in the formations of adducts with higher PEG contents and additionally, the PEG in these adducts strongly interact with the γCH_2 or the outer layer of the PGNA globule. This may be because of the effect of two combined factors: 1) the outer layer has more volume available for PEG than in the inner layer and, 2) the outer layer bound PEG are also stable since the higher molecular weight PGNA molecule has a large number of possible hydrophobic interaction/contact points (γ sites).

The opposite was observed for the lower molecular weight (17K) PGNA based adducts. These adducts had a lower PEG content, and 2-D NOESY NMR spectra

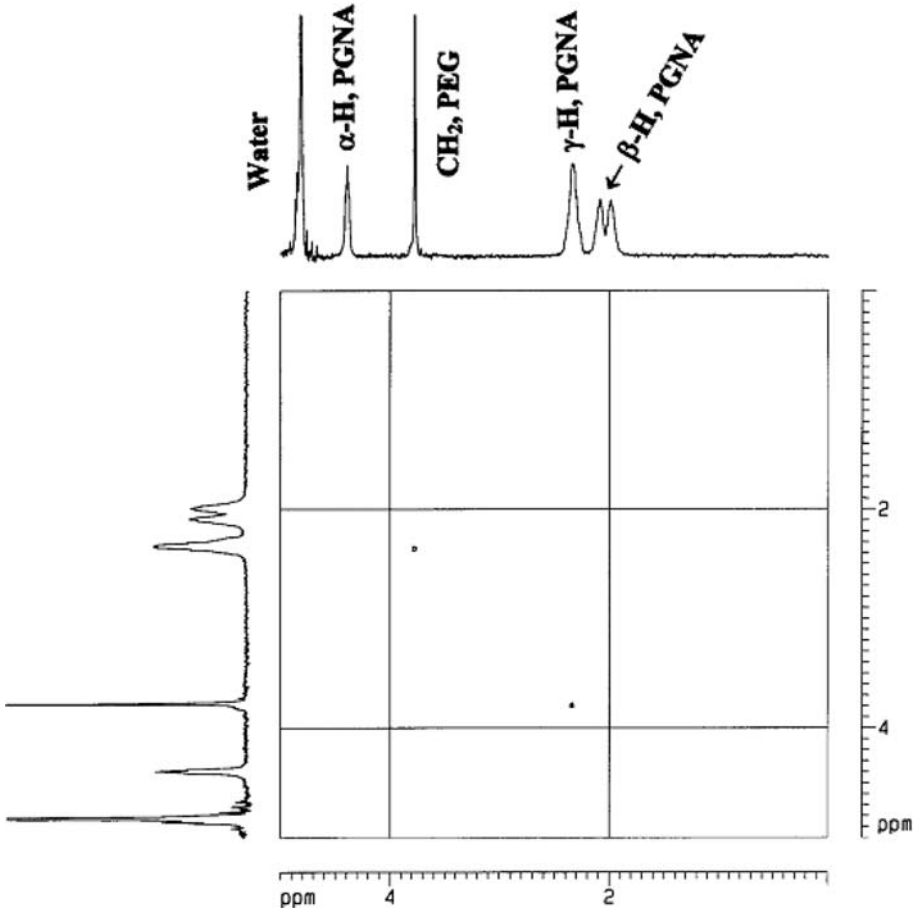

Figure 2. 2D-NOESY ^{1}H NMR spectrum of PGNA 32 K/PEG 10 K in D_2O at 5 mg/ml.

indicate a lower accumulation of PEG in γ CH_2 sites or the outer layer of the PGNA globule (Figure 3). This may be due to the loss of PEG from the outer layer during the purification process. The observation may be explained by considering that the lower molecular weight PGNA has fewer hydrophobic interaction/contact sites and this would translate to a reduction in number of hydrophobic contact points between the two components in the adduct. In adducts with the lower molecular weight PGNA, the PEG molecules are located closer to the PGNA backbone and hence, displays a more intense β correlation ($I_\beta > I_\gamma$).

Differential Scanning Calorimetry (DSC)

The DSC thermogram of the PGNA 32K/PEG 10K adduct is shown in Figure 4. The thermogram shows a melting temperature (T_m) at 70°C and a glass transition temperature (T_g) at 187°C. The T_g of PGNA 32 K has been reported to be a broad transition with a midpoint (T_g) at 216°C (13). In this blend, a sharp glass transition is observed at 187°C. Furthermore, the enthalpy of melting for the observed T_m is 2.8 J/g.

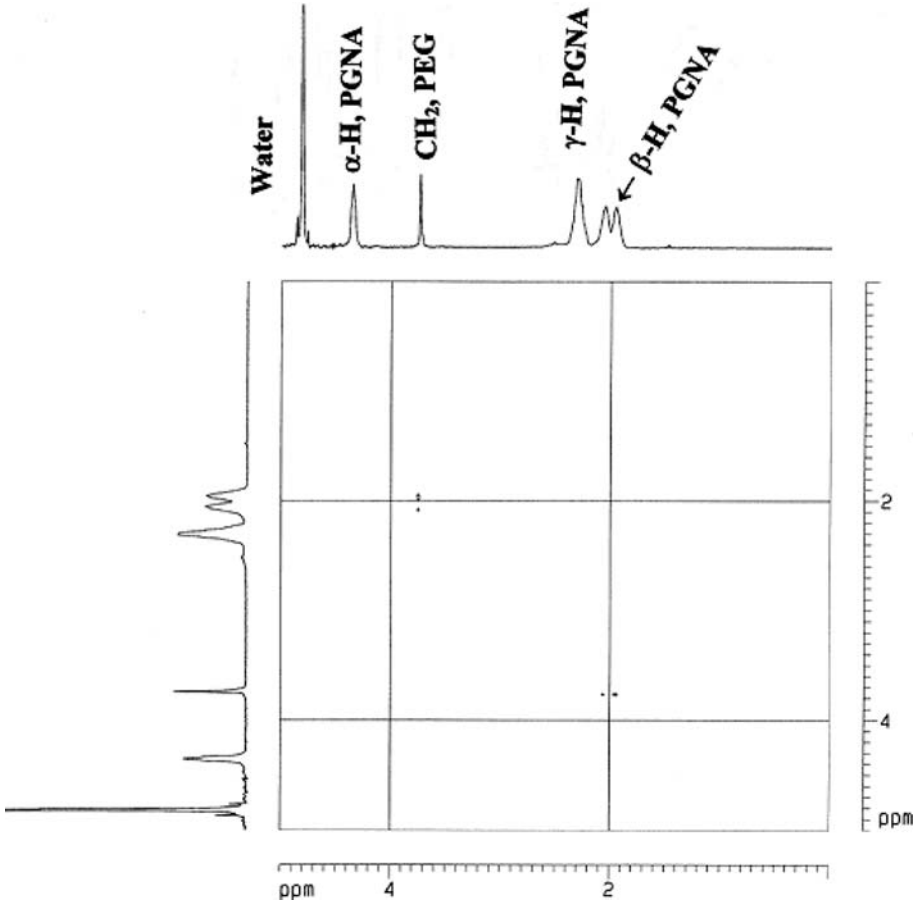

Figure 3. 2D-NOESY ^{1}H-NMR spectrum of PGNA 17 K/PEG 10 K in D_2O at 30 mg/ml.

This value is 70–80% lower than the enthalpy of melting of pure PEG 10 K. It may be concluded that the observed glass transition (T_g) at 187°C is most likely due to the formation of a compatible blend of PGNA and PEO.

CD Spectroscopy

The CD spectra of PGNA 17 K and PGNA 17 K/PEG 10 K in water are shown in Figure 5. Both CD spectra show only a negative peak at 239 nm indicative of a peptide with no higher structural order. Higher ordered peptide structures with α-helices and/or β-sheets exhibit both negative and positive peaks by CD spectra (22). The PGNA backbone is known to form a helical conformation when ion-dipole interactions are present in the blend system (14). Apparently, hydrophobic interactions between PEG and side groups of the PGNA chain alone are not sufficient to induce a helical conformation. The 2-D NOESY ^{1}H-NMR spectra suggest that the major interaction between the PGNA and the PEG is hydrophobic.

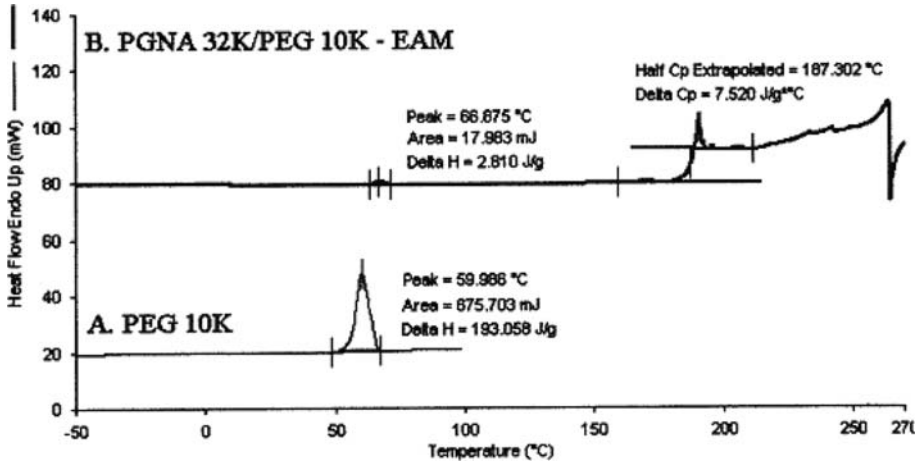

Figure 4. Solid state DSC thermograms of: A. PEG 10 K; B. PGNA 32 K/PEG 10 K.

Water Stability of the Non-covalent Adducts

Stability of non-covalent adducts in water can be monitored by measuring integrated peak areas (A) of the 2D NOESY ^{1}H-NMR spectra at a given time, since correlations for adduct (I) and free PEG (II) are widely separated:

I. PGNA-PEG correlations: A). 2.05–3.9 and 1.95–3.9 ppm for β site (Figure 3), and B) 2.15–3.9 ppm for γ site (Figure 2).
II. PEG--PEG correlation: 3.9–3.9 ppm.

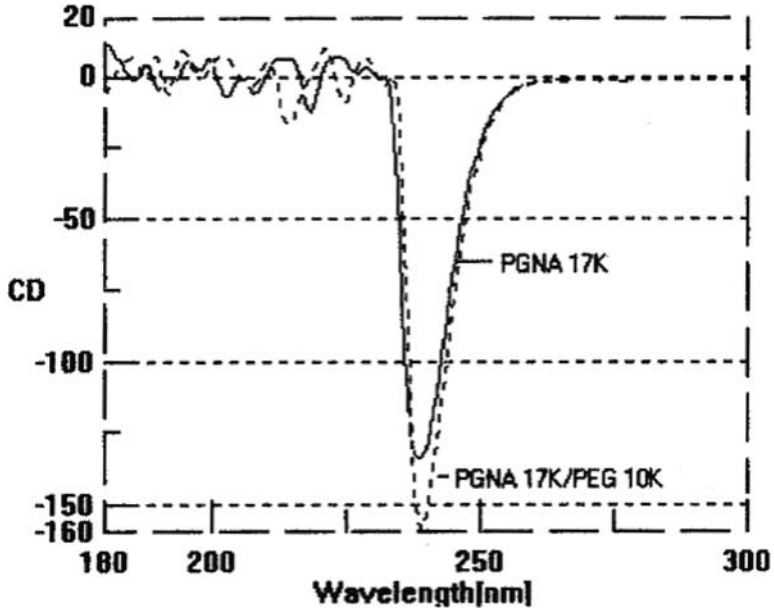

Figure 5. CD spectra of PGNA 17 K and PGNA 17 K/PEG 10 K in water.

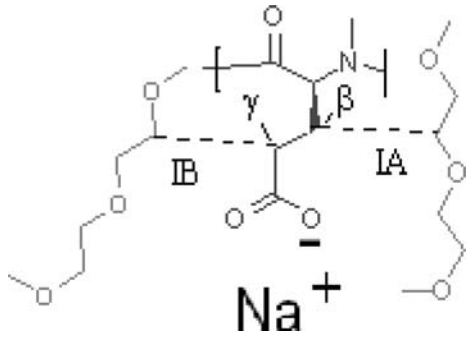

Figure 6. The two types of hydrophobic interaction between PGNA and PEG.

The two types of different hydrophobic interaction between the PGNA and PEG are shown in Figure 6. The dissociation of the non-covalent adducts into its components should lead to a decrease in $(A_\beta + A_\gamma)$ and an increase in A_{PEG}, or a decrease of the peak area ratio, $[(A_\beta + A_\gamma) \div A_{PEG}]$, where, A_β and A_γ represent peak areas of PGNA-PEG correlation (I) and A_{PEG} represents a peak area of free PEG (II). Stability studies for the PGNA 32 K/PEG 10 K and PGNA 17 K/PEG 10 K with $I_\gamma > I_\beta$ (in Figure 2) and $I_\beta > I_\gamma$ (in Figure 3) were carried out over 48 weeks at 5°C. The results are summarized in Figure 7. The results indicate that the PGNA 17 K/PEG 10 K adduct was stable in water for 35 weeks, whereas the PGNA 32 K/PEG 10 K adduct dissociated continuously and gradually into its components over the 48 week period. Room temperature water stability of two adducts with $I_\gamma > I_\beta$ and $I_\beta > I_\gamma$ are shown in Figure 8. The non-covalent adduct with $I_\beta > I_\gamma$ showed better water stability (>26 days).

Formation and dissociation of adducts can be described by an equilibrium process (Scheme 1). The rate constant of the forward reaction (k) for the equilibrium was calculated using an equation (Figure 9, Equation (2) established by Laider (23). The rate

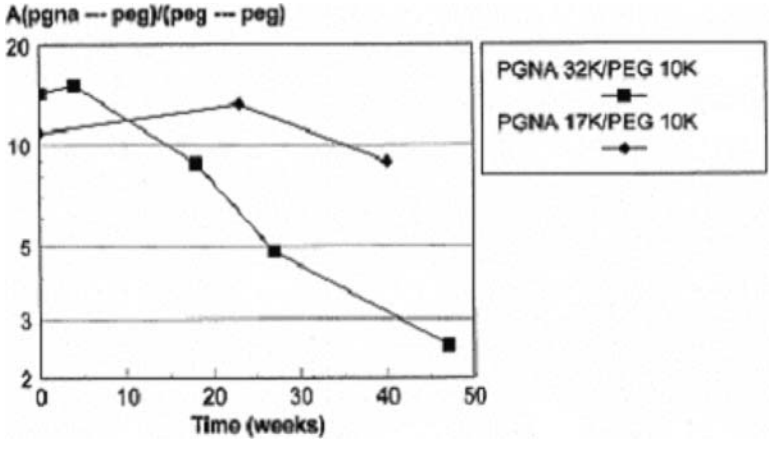

Figure 7. Room temperature 2D-NOESY ^{1}H-NMR peak area ratio, $[(A_\beta + A_\gamma) \div A_{PEG}]$ vs. incubation time (at 5°C) for non covalent adducts PGNA 32 K/PEG10 K-(5 mg/ml in D_2O) and PGNA 17 K/PEG 10 K (30 mg/ml in D_2O).

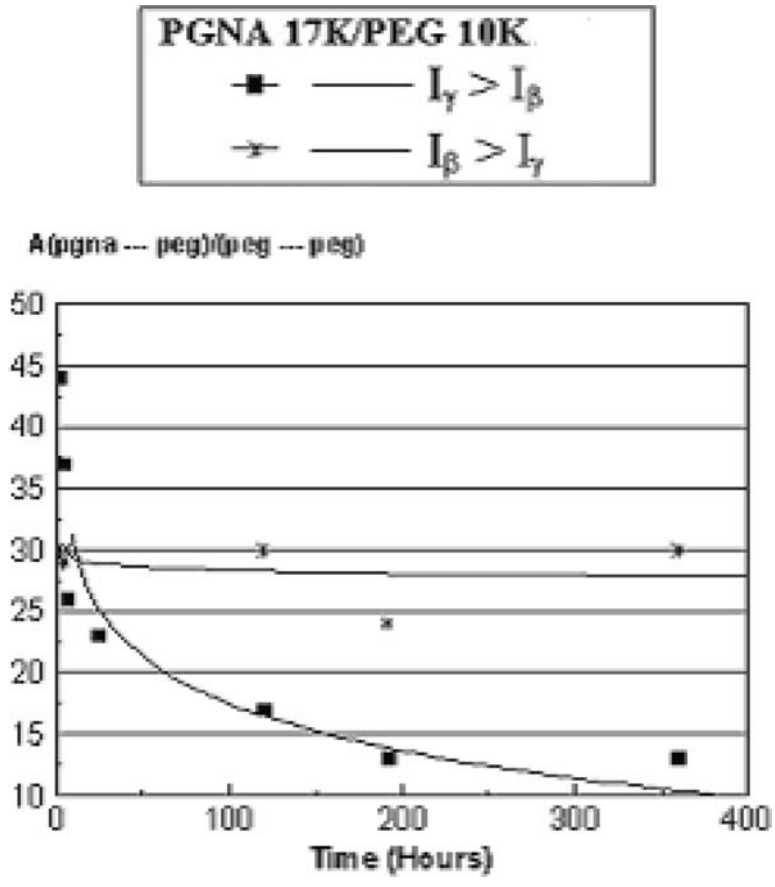

Figure 8. Room temperature 2D-NOESY ^{1}H-NMR peak area ratio, $[(A_\beta + A_\gamma) \div A_{PEG}]$ vs. incubation time (at room temperature) for non-covalent adducts PGNA/PEG.

constants of dissociation for the PGNA 32 K/PEG 10 K and PGNA 17 K/PEG 10 K adducts were $1.3 \times 10^{-2}\,h^{-1}$ and $7.2 \times 10^{-5}\,h^{-1}$, respectively. Clearly, the PGNA 17 K/PEG 10 K adducts with $I_\beta > I_\gamma$ is significantly more water stable than adducts with $I_\gamma > I_\beta$ i.e., it seems that the hydrophobic interaction between the β CH_2 of the PGNA and the CH_2 of the PEG is effective in preparing water stable non-covalent adducts.

Purified adduct is obtained after removal of excess PEG. Upon dilution, adducts may be forced to undergo following equilibrium releasing PEG from the adducts:

$$\text{PEG---PGNA---PEG or PEG---PGNA} \leftrightarrow \text{PEG---PGNA} + \text{PEG or PGNA} + \text{PEG}$$

Rate of Dissociation vs. Nature of Binding

The difference of water stability between the two adducts, $I_\gamma > I_\beta$ and $I_\beta > I_\gamma$, is interesting. For adducts having smaller amounts of PEG, e.g., PGNA 17 K based adducts having 1.7% (w/w) PEG, most of the PEG was bound to β sites. Total detachment of PEG from such sites at a given moment is less probable since β sites perhaps have limited segmental

PEG---PEG + PGNA $\leftrightarrow$ PEG---PGNA---PEG or PEG---PGNA + PEG

PEG + PEG $\rightarrow$ PEG---PEG (excess PEG)

Scheme 1. Formation and dissociation of non-covalent adducts.

motion. Non-covalent adducts based on PGNA 32 K (or $I_\gamma > I_\beta$) has PEG bound to both inner and outer layer of PGNA. However, the outer layer contains much more PEG than the inner layer, and the faster dissociation seen in this adduct was mainly due to loss of PEG from outer layer. Total detachment of PEG from such sites at a given moment is more probable, since γ sites have large segmental motions, i.e., large rotational movements. Furthermore, stability is dependent on the nature of binding rather than on the molecular weight of PGNA.

Conclusions

Water soluble and water stable non-covalent adducts of "a water-soluble peptide/a neutral water-soluble polymer" have been prepared. In aqueous solutions, adducts were stabilized via multi-prong hydrophobic interactions. Points of hydrophobic interactions were at

$$A \rightleftharpoons X + Y \quad (A = \text{adduct}, X = \text{PEG}, Y = \text{PGNA}) \quad \text{---- (1)}$$

$$\frac{x_e}{(2a_0 - x_e)} \ln \frac{a_0 x_e + x(a_0 - x_e)}{a_0(x_e - x)} = kt \quad \text{---- (2)}$$

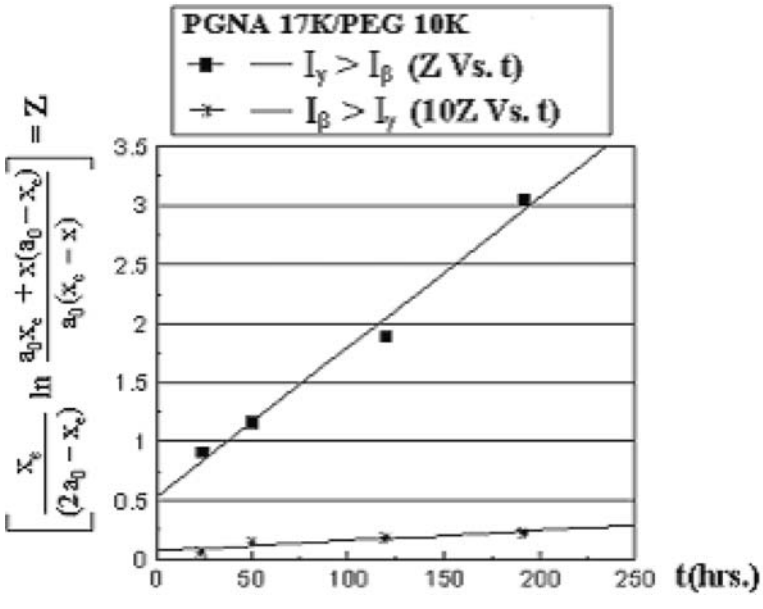

Figure 9. Time vs. Z plots for the dissociation curves given in Figure 8, where a_0—starting concentration of A, x_e—equilibrium concentration of X, and x—concentration of X at time t.

γ-CH_2 and β-CH_2 sites of PGNA and CH_2 groups of PEG. In these adducts, PEG binds to both the γ sites (outer layer) and β sites (inner layer) of PGNA. By selecting a sample that had much more PEG in the inner layer, it was possible to demonstrate prolong water stability ($>$26 days) at 25°C. Therefore, our observations raise the possibility of preparing water stable non-covalent adducts whose water stability may be tailored for a particular application e.g. controlled drug delivery.

Acknowledgements

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