ELSEVIER

International Journal of Pharmaceutics 127 (1996) 43–52

international journal of pharmaceutics

Adsorption of peptides to poly(D,L-lactide-co-glycolide): 2. Effect of solution properties on the adsorption

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Received 29 July 1994; revised 2 May 1995; accepted 4 May 1995

Abstract

The effect of pH, ionic strength, polarity and temperature on the adsorption of three peptides to poly(D,L-lactide-co-glycolide) (PLGA) was evaluated. Maximum adsorption was found near the pI of the peptide for salmon calcitonin (sCT), triptorelin (DP) and a peptide comprising the 8–22 amino acid portion of sCT. For sCT, almost no adsorption was observed at pH < 6 while there was complete depletion at pH 10. Increase in NaCl concentration enhanced the adsorption of sCT and DP. The dependency on solvent ionic strength and polarity suggested that hydrophobic interactions were playing an important role in the adsorption process. The net adsorption of sCT and DP was greater at 22°C than at 4°C or 37°C.

Keywords: Peptide-polymer interaction; Salmon calcitonin; Adsorption; Poly(D,L-lactide-co-glycolide); Desorbtion

1. Introduction

Adsorption studies of proteins on to biomaterials have generally been conducted with serum proteins (such as albumin and fibrinogen) under physiological conditions (37°C, 0.15 M NaCl, pH 7.4). Such studies have suggested that both electrostatic and hydrophobic interactions are involved in the adsorption of a protein onto a surface (Elwing et al., 1987; Muramatzu and Kondo, 1992). The parameters which influence these interactions include pH, ionic strength, solvent polarity and temperature.

The effect of pH on protein adsorption has been studied and generally the maximum adsorption occurs at or near the pI of the protein (Norde, 1986; Luey et al., 1991). The degree to which ionic strength affects protein adsorption is greatly dependent on the role of electrostatic interactions on the adsorption driving force (Luey et al., 1991). The degree to which pH affects the adsorption has been found to be determined by the conformational stability of the particular adsorbate molecule. Norde and co-workers have observed that the adsorption of bovine pancreas ribonuclease (RNase) to negatively charged sur-

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SSDI 0378-5173(95)04112-N

faces was essentially independent on pH, while that for human plasma albumin (HPA) varied dramatically (Norde, 1986). They have reasoned these observations based on the relatively higher degree of conformational flexibility of HPA as compared to RNase, which allows it to change structure as conditions in the solution change. Studies with other proteins also suggest the contribution of conformational flexibility on adsorption to solid surfaces. Immunoglobulin G's exhibit various degrees of repulsion in the $F(ab')_2$ domain by changing pH, while for triple-helical collagen molecules that are structurally rigid, pH and ionic strength had no effect on the adsorptive behaviour (Horbett and Brash, 1987). In general, it has been found that, for those conditions leading to a less stable protein conformation in solution, an increased adsorption will be observed (Dillamn and Miller, 1973; Norde, 1986; Horbett and Brash, 1987; Young et al., 1988). Peptides which are flexible can alter their structure more easily to fit adsorbent sites than those that are rigid. In an organic solvent, peptide molecules are more likely to adopt a helical or pleated-sheet structure via intramolecular hydrogen bonding (Stryer, 1981; Horbett and Brash, 1987), and are less likely to fold into a compact structure as generally occurs in a water rich medium. When the peptide molecules are interacting with a solid surface in aqueous medium, as an alternative for intramolecular hydrophobic interaction, apolar parts of the peptide may be exposed to the sorbent surface and remain shielded from water.

Wahlgren and Arnebrant have studied temperature effects on the adsorptive behaviour of a milk protein, β-lactoglobulin. A substantial increase in the amount adsorbed with faster kinetics when the temperature was increased to the point where protein denaturation started. The temperature effect was an indication that hydrophobic interactions were contributing to the adsorption process (Dillamn and Miller, 1973; Soderquist and Walton, 1980). Occasionally, decreased adsorption by increasing temperature has been observed (Mitra and Chattorji, 1978). This is probably a stability effect (as well as conformational change) in some protein solutions whereby at lower temperature the protein tends to 'salt out' and deposit at an interface. Macromolecules can have multiple contact points with the adsorbent, and irreversible adsorption is likely due to the difficulty of simultaneous dissociation of these contacts from the sorbent surface (Elwing et al., 1987; Nygren and Stenberg, 1988; Stuart et al., 1984a; Stuart et al., 1984b). It has been reported that some negatively-charged proteins are not repelled from negatively-charged surfaces (Norde, 1986). The reasons were generally thought to be due to conformational adjustment of the proteins themselves.

The non-specific adsorption of proteins to interfaces have generally been attributed to the entropic effect, commonly termed as hydrophobic interactions (Norde, 1986). Even when the adsorption was often found to be greater with more hydrophobic surfaces (Brynda et al., 1984; Luey et al., 1991), there is no conclusive correlation between the hydrophobicity of proteins and adsorption. It has been suggested that hydrophobic interactions can be classified as long range Lifshitz-van der Waals (LW) interactions (of the London + Keesom + Debye varieties) and short ranged (SR) interactions caused by hydrogen bonds (van Oss et al., 1988). It has also been demonstrated that the interfacial forces, the hydrophobic interactions between proteins and hydrophobic surfaces immersed in aqueous media, are the combination of LW and SR interactions which could be separated via sophisticated contact angle measurements (van Oss and Good, 1984).

The structure of the peptide, especially the rigidity of the peptide molecules, can affect the surface interaction with solids due to conformational modification of the peptide on the solid surface. Horbett and Brash, 1987 have stated that more hydrophobic and less soluble proteins may be more surface active, which seems to be a general rule but not necessarily correct if one considers hydrophobic interactions are actually the sum of LW and SR forces, with the latter causing the initial contact between adsorbate and adsorbent (van Oss and Good, 1984). Some studies showed that more hydrophobic peptides interacted to a greater extent at the interfaces if the

molecular weights of peptides were similar (Ruzgas et al., 1992).

The objective of this study was to evaluate those factors which have been shown to influence the extent of adsorption for peptide adsorption to PLGA, namely, pH, salt, solvent polarity and temperature.

2. Materials and methods

2.1. Materials

Polymer: poly(D,L-lactide-co-glycolide; 50:50) (PLGA) 34 000 MW (Boehringer Ingelheim, Germany).

Peptide: the purity of the peptides were all > 97%, therefore the peptides were used without further purification. Salmon Calcitonin (sCT) (Bachem Inc., Torrance, CA), Salmon Calcitonin 8–22 residues (CT15) (MSAF, University of Kentucky, Lexington, KY), LHRH analogue Decapeptyl (DP) and somatostatin analogue RC160 (Debiopharm, Lausanne, Switzerland).

Buffer solution: phosphate buffer was prepared with sodium phosphate dibasic heptahydrate and sodium phosphate monobasic monohydrate and, if necessary, the pH was adjusted with dilute phosphoric acid and sodium hydroxide (all from Aldrich Chemical Co., Milwaukee, WI).

Other materials: polypropylene vials (1.5 ml capacity), acetonitrile and methanol, HPLC grade, were from Fisher Scientific Co. (Fair Lawn, NJ). Sodium chloride was from Sigma Chemical Co. (St. Louis, MO). Ultrafiltered water from a Milli-Q water system was used (Millipore, Bedford, MA).

2.2. Methods

2.2.1. Preparation and analysis of peptide solution

A peptide solution of the desired concentration was prepared fresh by proper dilution from a 1 mg/ml stock. The stock solution was prepared by dissolving 10 mg of the peptide in the adsorption medium at a final volume of 10 ml. Analysis of peptides was by reversed phase HPLC as described previously (Tsai et al., 1996).

2.2.2. Adsorption study

The adsorption procedure was the same as that described earlier (Tsai et al., 1996). In brief, the extent of adsorption was obtained by a solution depletion technique. The incubation time for adsorption in this study was 4 h unless otherwise stated. Bound peptide was calculated from the difference between the initial and free peptide concentrations. Peptide and polymer controls were carried out along with the adsorption samples. The effect of pH was studied over the range from the acidic region to the pI of the peptide. The pH of each 0.1 M phosphate buffer solution was prepared by combining the required amounts of 0.1 M sodium phosphate monobasic and dibasic solutions. The pH values from 4.0 to 10.0 were prepared and at least 4 values were tested on an individual peptide. The effect of salt was studied by using 0, 5 and 10% w/v NaCl in water. Methanol solutions of 0, 10 and 20% v/v in water were prepared to test the co-solvent effect. The experiments were performed at 4, 22 and 37°C.

2.2.3. Desorption

The adsorption conditions for preparing samples were as follows:sCT: 1 ml peptide solution (C_o = 1000 μg/ml) with 20 mg of PLGA; DP: 1 ml peptide solution (C_o = 200 μg/ml) with 20 mg of PLGA.

After treatment in 0.1 M phosphate buffer, pH 7.4 for 4 h, the peptide-adsorbed particles were separated from the medium, gently washed with fresh buffer, centrifuged and freeze dried. The amount of peptide on the polymer was calculated by subtraction from the initial value as well as from an extraction procedure. The extraction procedure was as follows: a weighed amount of the particles were transferred to a polypropylene tube and 1 ml of 50% acetonitrile in 0.01 M phosphate buffer (pH 7.4) was added. The suspension was sonicated for 15 min, centrifuged and the supernatant was collected. This procedure was repeated twice with 0.5 ml extraction vehicle. The peptide content in the 2 ml of collected extracts was determined by HPLC.

Desorption was performed in 1.5 ml polypropylene vials in duplicate in the medium in which the sample was initially treated for the adsorption. In

each vial, 5 mg of the sample, prepared by the procedure mentioned above, were suspended in 1 ml of the desorption medium and placed on a tube rotator with a constant rotation speed of 18 cycles/min. To compare the parameters studied for the adsorption of peptides, desorption was performed at different pH values, salt contents and temperature. More conditions were evaluated on the sCT samples than the DP samples due to the simplicity of analyzing the concentrations desorbed. Where readsorption was observed, desorption was carried out by complete replacement of the desorption medium upon each analysis.

3. Results and discussion

3.1. Effect of pH

Fig. 1 shows the effect of pH on the adsorption of sCT, DP and CT15 to PLGA, respectively. Fig. 1a shows that as pH was increased from 4 to 10, sCT adsorption increased dramatically between pH 6 and 10. sCT has a pI around 10.2 and at pH below this value, the peptide is positively charged. Higher net charge on the peptide molecules could result in repulsion within and between peptides that would demand larger interspace to overcome the repulsive force. At the pI of the peptide, molecules with a zero net charge could approach each other more closely and form a more compact conformation resulting in more effective adsorption. Evaluation above pH 10 was not possible for sCT due to the solubility limit of the peptide.

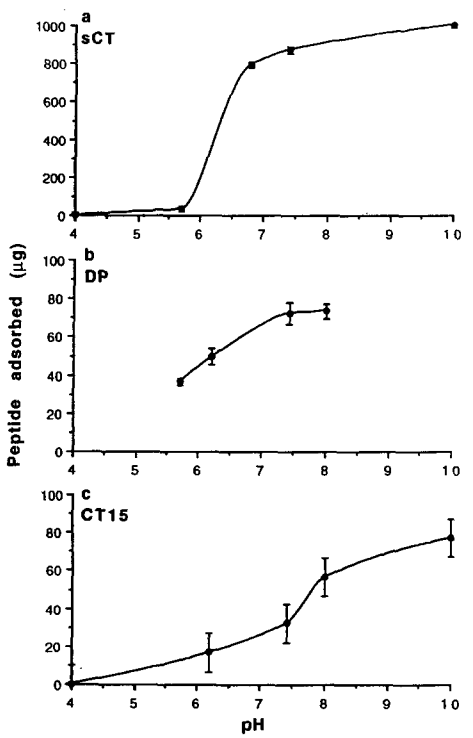
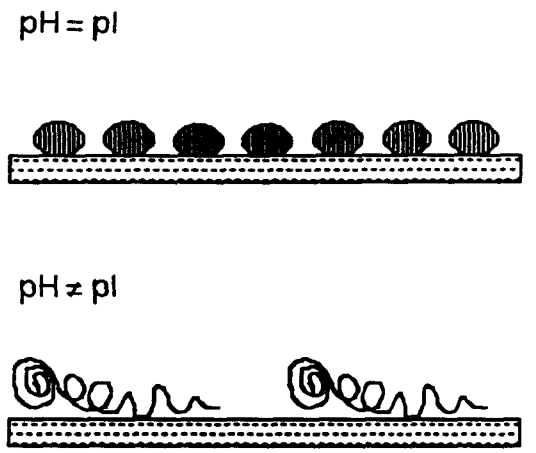


Fig. 1. Effect of pH on the adsorption of peptides to 10 mg PLGA. (a) sCT, C_o = 1 mg/ml; (b) DP, C_o = 0.2 mg/ml; (c) CT15, C_o = 1 mg/ml. Data for each point were averaged from triplicate samples.

The adsorption for Decapeptyl and CT15 showed similar pattern as that for sCT as shown in Fig. 1b and Fig. 1c. Decapeptyl has a pI around 7.6, and maximum adsorption was found in the 7.4–8.0 pH range. CT15 has a calculated pI around 9.4 and the pH dependency using C_o = 1 mg/ml was similar to that for sCT even though the extent of adsorption was much lower than with sCT. The extent of maximal adsorption observed for CT15 was similar to DP with C_o = 0.2 mg/ml. RC160 was not soluble in the phosphate buffer system used for the other three peptides. Adsorption of RC160 in water (pH around 5.7 due to the acetate salt) showed no adsorption. Since the pI of RC160 is in the basic region, RC160 is available as the acetate salt to provide better solubility.

Less repulsive forces between adsorbate molecules resulting in compact packing of the adsorbates and possible change of adsorbate conformation as a result of pH are the reasons for maximal adsorption around the pI. Scheme 1 presents explanation of the pH effect (Norde, 1986). As depicted in the scheme, when the pH equals the pI, the adsorbate molecules are presented in a compact form; whereas away from this pH, they are in a loose form to overcome its intramolecular charge repulsion. This has been shown to occur with fibrinogen by comparing the monolayer formation using scanning electron microscopy (SEM) (Nygren and Stenberg, 1988). Since peptides are much smaller molecules compared to fibrinogen and the polymer was in powder form, observation by SEM was not successful. The pep-



Scheme 1. Hypothetical scheme of peptide conformation at and away from pI.

tides studied showed decreased solubility as the pH was increased from acidic region towards the pI of the peptides. Horbett and Brash, 1987 suggested that around the pI, adsorbates are in a less soluble environment which leads to greater adsorption (Kaelble and Moacanin, 1977).

3.2. Effect of salt

The effect of NaCl on the adsorption of the peptides to PLGA is shown in Fig. 2. Adsorption increased as the salt concentration was increased. The slopes of the (a) and (b) regression lines were similar indicating that the salt effect on sCT and DP was similar. Although the results for CT15 were not conclusive due to the lower adsorption and the variation between samples, it appears salt had a similar effect. RC160 did not show any detectable adsorption to PLGA under the same conditions. However, RC160 with C_0 = 1 mg/ml was found to adsorb 20% in 10% NaCl solution after 8 h. Only intact peaks for all peptide controls were observed on the HPLC chromatograms and, there was < 5% difference in concentration after the treatment.

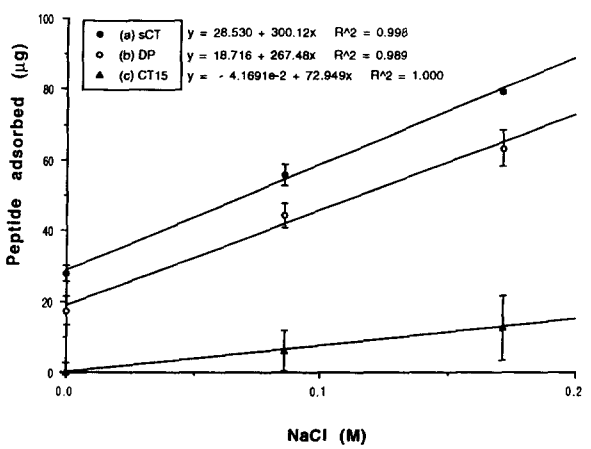
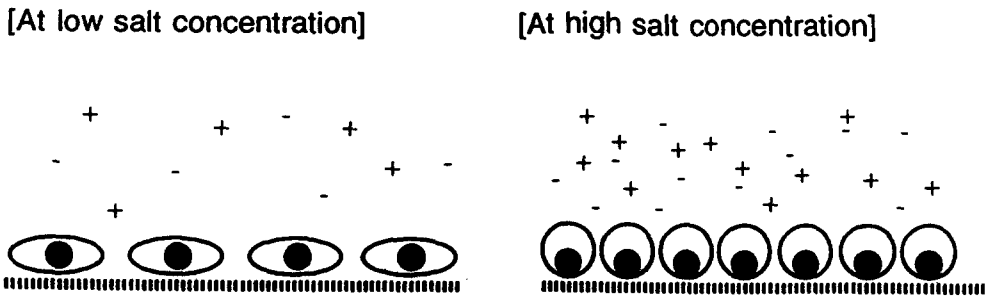


Fig. 2. Effect of NaCl on the adsorption of (a) sCT, (b) DP and (c) CT15 to 10 mg PLGA. C_0 = 100 µg/ml. Data for each point were averaged from triplicate samples.

An explanation for the salt effect is that the existence of ions provides a less desirable environment for hydrophobic regions of the peptide. Under this condition, the peptide's hydrophobic region has to bury further into the interior of the molecule to be away from the medium. This structural arrangement results in a more compact molecule than that in pure water as illustrated in Scheme 2. The hydrophobic regions of the peptide (represented by the shaded area) are squeezed from the aqueous phase and sometimes reside on the polymer surface to avoid contact with water. As the salt concentration increases, the escaping tendency of hydrophobic regions from the medium becomes stronger; meanwhile, the hydrophilic parts of the peptide become less repulsive to each other (if oppositely charged) due to the shielding effect of the ions in the medium. Therefore, at higher salt content, a more compact structure of the peptide molecule is formed.

Another possible reason for the salt effect can be based on solubility (Kaelble and Moacanin, 1977). If adding salt results in a salting-out effect, the peptide could reside at the interface for better stability. However, if the presence of salt helps to stabilize or increase the solubility of the peptide (the salting-in effect), a reverse type of salt effect on the adsorption would be observed. The peptides studied were easier to solubilize in water than in the NaCl solutions, therefore the results were more consistent with that of a salting-out effect. As the salt concentration increased, it appeared the more stable arrangement for the peptide molecules would be to associate with the polymer surface. This has been an accepted explanation for proteins even though experimental verification has been limited.



Scheme 2. Hypothetical scheme of peptide conformation at low and high salt concentrations.

The effect of buffer concentration on the adsorption of sCT is shown in Fig. 3. There was a linear dependency similar to that with NaCl. However, the slope from the regression line was much different from that with NaCl. The pH difference in aqueous NaCl and the buffered condition may also play a role. The pH of sCT in aqueous NaCl solution was around 5.6 since acetate buffer was added when formulating the dried powder to increase the solubility. In the presence of a buffer, the effect of acetate is diminished and the pH of the solution is controlled by the buffer. The peptide concentration used to evaluate the phosphate effect was 10 times higher than that used for NaCl. Since adsorption of sCT was enhanced at higher peptide concentration and higher pH, the higher adsorption with phosphate could be attributed to these conditions.

3.3. Effect of solvent polarity on the adsorption

Fig. 4 shows the effect of polarity on the adsorption of sCT and DP. As methanol concentration was increased, the adsorption of sCT to PLGA reduced almost linearly. With DP, there seemed to be a similar trend but there was no statistical difference between the three methanol concentrations. The relationship between dielectric constant and sCT adsorption is shown in Table 1. In less polar medium, hydrophobic regions of the peptide molecule are less likely to escape from the medium, and they could be more freely distributed in the molecule. The adsorption in methanol can also be attributed to the more ordered conformation of the peptide molecules. The secondary structure of sCT in aqueous solution has been found to be enhanced by the addi-

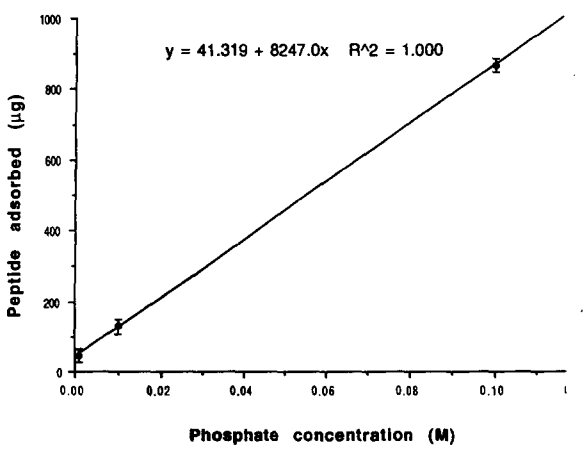


Fig. 3. Effect of phosphate salt on the adsorption of sCT to 10 mg PLGA. C_o = 1 mg/ml. Data for each point were averaged from triplicate samples.

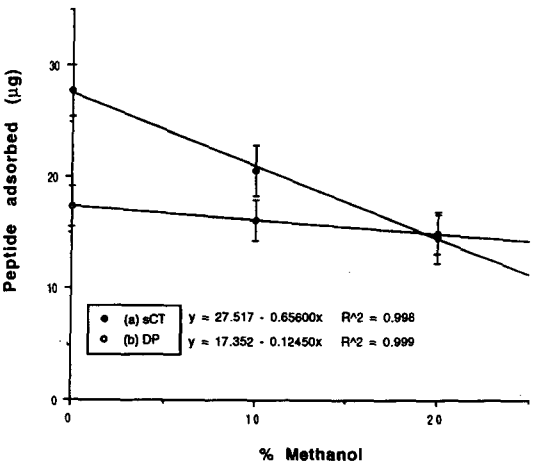


Fig. 4. Effect of methanol concentration on the adsorption of (a) sCT, (b) DP and (c) CT15 to 10 mg PLGA. C_o = 100 µg/ml. Data for each point were averaged from triplicate samples.

Table 1
Relationship between dielectric constant and sCT adsorbed >

% Methanol in water	Dielectric constant	Adsorbed (μg/10 mg)
0	80.0	27.8 ± 2.3
10	75.3	20.6 ± 1.6
20	70.6	14.6 ± 1.7

Experiments were carried out in triplicate for 4 h at 22°C.

tion of methanol (Motta et al., 1989). In the presence of a structure enhancing agent, the peptide's ability or propensity to associate at the polymer surface is reduced.

3.4. Effect of temperature on the adsorption

The temperature effect on the adsorption of sCT and DP is shown in Fig. 5. In both cases, the net adsorption to the polymer, PLGA, increased

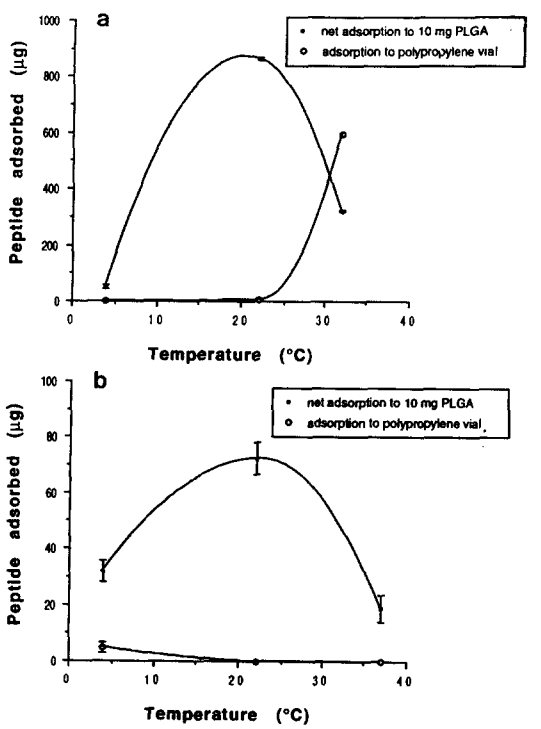


Fig. 5. Effect of temperature on the adsorption of peptides to 10 mg PLGA. (a) sCT, C_o = 1 mg/ml (b) DP, C_o = 200 μg/ml. Data for each point were averaged from triplicate samples.

when the temperature was increased from 4°C to 22°C. The net adsorption decreased when the temperature was further increased. The concentration of a sCT control at 32°C for 4 h was significantly reduced, probably due to preferential adsorption to the polypropylene vial at the higher temperature. It was found that the sCT controls for the other two temperature conditions were stable with a reduction of < 3% .The interaction between sCT and the polypropylene surface could probably be driven by hydrophobic interactions which became more pronounced at higher temperature. Unlike that for sCT, DP did not adsorb to the container at higher temperature although there was some reduction of DP at 4°C in the control. It was found that when the DP solution was subjected to lower temperatures (22°C or 4°C) without stirring, insoluble precipitates formed within 4 h (more at 4°C than at 22°C). However, no precipitates were found when the solution was kept at 37°C and for the DP controls which were agitated. It is a rather interesting phenomenon that the peptide appears to be more stable under agitation and at higher temperature. The stability phenomenon observed with DP was contrary to general peptide stability where higher temperature and agitation tend to destabilize peptides. The aggregated DP precipitates could not be redissolved even by increasing the temperature to 37°C. This could be the same phenomenon that Sanders et al., 1986, 1990 observed with another LHRH analogue stating a decapeptide (nafarelin) has been (indirectly) observed to display secondary conformational structure, in that it forms an ordered structure of liquid crystals, analogous to β-sheet formation, in a suitable aqueous environment, in aqueous solution, particularly in the presence of electrolytes, birefrigence is observed, and this ordering in solution exhibits itself in the extreme case as gelation'. The effect of temperature is probably due to the combination of hydrophobic interactions and solubility (or stability).

Fig. 6 shows the effect of temperature on DP adsorption in 0.1 M phosphate buffer, pH 5.7 and 0.1 M Tris buffer, pH 7.4 . The adsorption was the highest at 22°C and the lowest at 37°C at pH 5.7. The same temperature effect on the adsorption was found at pH 7.4 Tris buffer. The reduc-

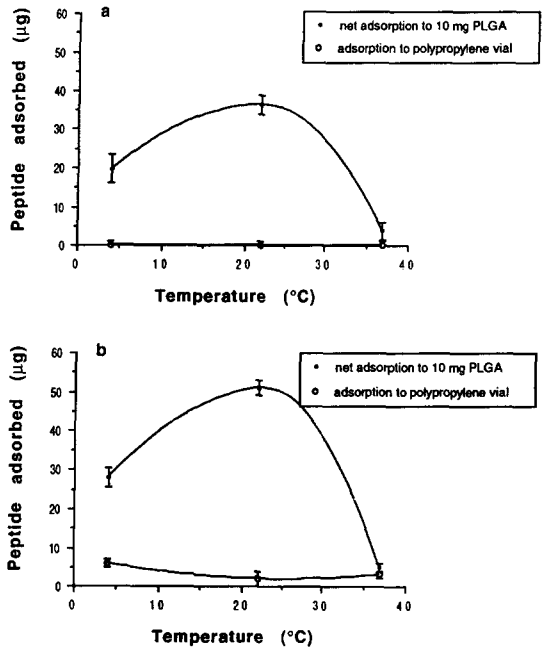


Fig. 6. Effect of temperature on the adsorption of DP to 10 mg PLGA in (a) 0.1 M phosphate buffer, pH 5.7 and (b) 0.1 M Tris buffer, pH 7.4. C_o = 200 μg/ml. Data for each point were averaged from triplicate samples.

tion of peptide in the control at pH 5.7 was low despite the differences in temperature. Comparing the two phosphate buffer systems, DP was easier to dissolve in the pH 5.7 solution while it was very difficult to dissolve in the pH 7.4 phosphate and Tris buffers. The adsorption at pH 5.7 was less than that at pH 7.4 suggesting that the stability and solubility of DP in a medium would affect the amount of adsorption.

As mentioned in the introduction, hydrophobic interactions have been shown to be a combination of SR and LW forces and the SR forces are attributed to the hydrogen bonds (van Oss and Good, 1984). It is possible that the interactions between different peptides and the polymer involved various contribution of SR and LW forces. From the trend of the temperature effect, the interaction between sCT and the polypropylene vial could be due to LW forces which occurred at the higher temperature. However, it is more likely that the interaction between sCT and PLGA involved a greater contribution of SR forces. At pH 7.4 there was strong indication of competitive binding at 32°C between PLGA and polypropylene surfaces. For DP, the adsorption was less affected by the presence of the polypropylene surface and the trend of temperature effect was more related to the solubility and stability of the peptide at different temperature conditions.

3.5. Desorption study

Released peptide was found to readsorb to the polymer. Therefore, frequent medium replacement was necessary to minimize the readsorption effect and allow for better evaluation of the desorption pattern.

Fig. 7 shows the dependency of the influencing factors on the desorption of sCT from a preadsorbed state. Water resulted in the greatest desorption with regards to the amount and the rate. Over 60% of the adsorbed peptide was desorbed in water in the first hour and over 90% desorbed after 2 h. Comparing the desorption in 0.1 M and 0.01 M phosphate buffer at pH 7.4, the desorption was faster and greater in the 0.01 M medium. Between the 0.1 M solutions, desorption was the greatest and fastest at pH 2 followed by pH 7.4 and pH 8.0, respectively. The desorption pattern therefore followed the adsorption trend. In the less adsorbed medium, the desorption was greater. Fig. 8 shows the desorption of DP preadsorbed in 0.1 M phosphate buffer, pH 7.4. Between the 0.1

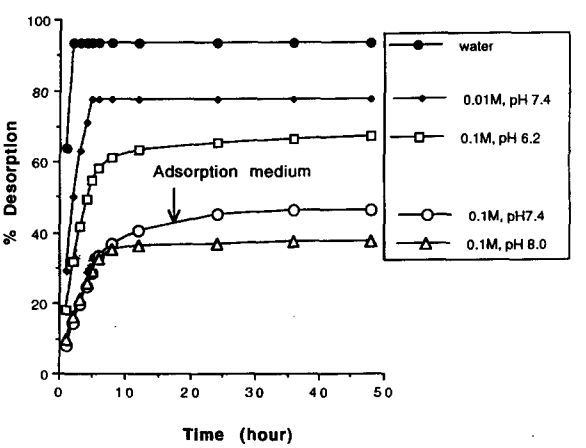


Fig. 7. Desorption of sCT from 5 mg sCT-adsorbed PLGA. C_o = 237 μg. One millilitre of the medium replaced at every sampling time. Data for each point from duplicate samples.

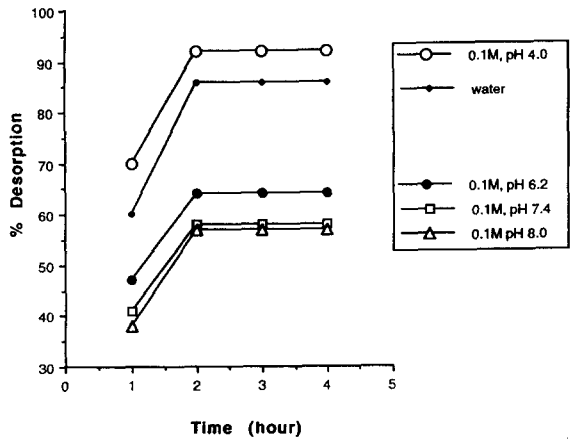


Fig. 8. Desorption of DP from 5 mg sCT-adsorbed PLGA. C_o = 28 μg. One millilitre of the medium replaced at every sampling time. Data for each point from duplicate samples.

M phosphate buffer solutions, the effect of pH on the desorption was the same as that with the desorption of sCT. Comparing the desorption in water and pH 4.0 phosphate buffer, the latter medium desorbed more. Even though water is with less salt when compared to the buffer, the pH effect appeared to over-ride the salt effect between these two conditions.

4. Summary and conclusion

The maximal adsorption for sCT, DP and CT15 was found to be around the pI of the peptide. For sCT, almost no adsorption was observed at pH < 6 and complete depletion occurred at pH 10. Greater adsorption may be attributed to less soluble environment around the pI. Increase of NaCl content in the solution was found to enhance the adsorption of sCT and DP probably due to a salting-out effect, decreasing solubility and enhancing association with the polymer. The same concentration dependency was found when the adsorption of sCT was performed in phosphate buffer but the adsorption was higher than that in NaCl, probably due to the higher pH of the phosphate buffer. The adsorption of sCT was reduced by the addition of methanol to reduce solvent polarity probably due to a more ordered peptide conformation and less desire to associate with the polymer. The dependency on salt and solvent polarity suggested that hydrophobic interactions were playing an important role in the adsorption.

The net adsorption of sCT and DP to PLGA was the greatest at 22°C among the three temperature conditions studied. The concentration of sCT control at 32°C for 4 h was significantly reduced, possibly due to preferential adsorption to the polypropylene vial at the higher temperature. Unlike that for sCT, DP did not adsorb to the container at higher temperature.

The desorption of sCT and DP from a preadsorbed state followed the adsorption trend. The rate and amount of desorption were greater in the medium which was less favored for adsorption. Adsorption of peptide in solution to the polymer surface can be applied to the delivery of peptides. A more interesting area currently under investigation is the encapsulation into a biodegradable polymeric system. Peptides and polymers in most controlled delivery formulations often involve an initial contact in an organic solvent for dissolving the polymer with the peptide in another aqueous phase or organic phase. Understanding the interactions between the two in these solvent systems can help in the design of the formulation.

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