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*Report*

# Adsorption of Salmon Calcitonin to PLGA Microspheres

Sema Calis,[1] Ramasubbu Jeyanthi,[2] Tsuimin Tsai,[3] Rahul C. Mehta, and Patrick P. DeLuca[4,5]

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*Purpose.* The interaction of salmon calcitonin (sCT) and poly (d,l-laetide-co-glycolide) was detected during preparation and evaluation of microspheres. The purpose of this study was to quantitate the extent and nature of the interaction. *Methods.* Blank microspheres were prepared by an aqueous emulsification solvent extraction technique. Adsorption studies were carried out at six concentrations of sCT and three concentrations of microspheres. Adsorption^isotherms were constructed using the Langmuir and Freundlich treatments. *Results.* Adsorption at I mg/ml sCT concentration resulted in almost complete depletion of the peplide from the adsorption medium with the time to reach maximum adsorption decreasing with increasing microsphere concentration. At sCT concentrations below 100 ^ig/ml, a true equilibrium occurred in 1 hour or less while at higher concentrations (up to 350 p-g/ml), a transient equilibrium was reached in 1 to 2 hours, followed by further adsorption of the peptide. The adsorption followed the Lang.-nuir isotherm at concentrations below 200 (Jig/mi, indicating formation of a monolayer. Multilayer interaction, described by (he Freundlich isotherm, occurred at higher concentrations and resulted in complete depletion of sCT from the adsorption medium. The affinity constant during monolayer formation was 0.09 and the plateau surface concentration was 5.1 Hg/mg. The multilayer peptide-pcplide adsorption showed a iower affinity (0.025) but higher capacity (24 p.g/mg» than the monolayer peptide-polymer adsorption. *Conclusions.* The results show that poly (d.l-lactide-co-glycolide) microspheres have a high adsorption capacity for sCT which must be considered in formulating a controlled delivery product of this peplide.

**KEY WORDS:** peptide-polymer interaction; salmon calcitonin; adsorption kinetics; adsorption isotherms: PLGA.

## INTRODUCTION

The interaction of peplides and proteins at interfaces is of great biological, medical and technical significance. Due to the advances in biotechnology and genetic engineering, peptides and proteins have become very important therapeutic agents. Adsorption of proteins to polymeric surfaces has been reported (1-3) for medical devices in which the primary concern was the adsorption of proteins to the implanted polymeric materials (4-8) Adsorption of peptides to polymer surfaces appears to involve a series of complex interactions which are neither easily described nor accurately predicted. sCT is a cyclic 32 amino acid peptide that is synthesized and stored by the parafollicular cells of thyroid, parathyroid and thymus glands. Since its first isolation, calcitonins have been obtained from many different species, however, fish calcitonins have been found to be more potent than mammalian ones (9). sCT has a 1-7 disulfide bridge followed by a [8-22] helix and a [23-32], p-sheet or random coil (10) structure. Its principal pharmacological action is the inhibition of bone resorption.

Recently, synthetic polymers such as polylactic acid (PLA), polyglycolic acid (PGA) and co-polymers of lactic and glycolic acid have gained considerable importance as carriers for controlled delivery of drugs and biologicals (11-13). These polymers are currently used in surgical applications because of their biocompatibility and biodegradability. The nature of the adsorption of therapeutic peptides on the surface of these polymers is of considerable importance in the design of drug delivery systems and in determining whether the incorporation and the release of peptide can be modulated by the peptide-polymer interaction.

Interaction of sCT with hydrophobic biodegradable polymers of lactic and glycolic acids has been evaluated in our laboratories to quantitate the binding parameters. The molar binding ratios of sCT to powdered polymers in aqueous solutions were 0.3:1 for PLA, 0.7:1 for PGA and 2:1 for the copolymer: These results indicated an extensive binding of sCT to the copolymer when compared to PLA and PGA (14-15). sCT was also found to adsorb strongly to PLA, PGA and the copolymer when the polymers were in solution, powder or microsphere form with the highest uptake occurring when both peptide and polymer were in solution (16). The importance of the adsorption phenomenon was evident in the preparation of microspheres. Suspension of the peptide in the polymer solution, which is expected to produce no interaction, resulted in microspheres with less than 5% entrapment efficiency. On the other hand. a solution of peptide and polymer produced microspheres with 65 to 90% entrapment efficiency due to the interaction between peptide and polymer prior to preparation of microspheres. The objectives of the present study were to evaluate the effect of polymer concentration (amount of microspheres) and peptide (sCT) concentration on the kinetics of sCT adsorption to poly (d.l-lactide-co-glycolide) (PLGA) microspheres and to determine the type of adsorption using the Langmuir and Freundlich models.

## MATERIALS AND METHODS

### Materials

PLGA (50/50), Resomer® RG503, mw 34000 was obtained from Boehringer Ingelheim, Germany, and sCT was obtained from Bachem Inc., Torrance, CA. All other chemicals used were of analytical reagent grade or better. Polypropylene vials used for the adsorption studies were obtained from Fisher Scientific, St. Louis, MO.

' Present address: Faculty of Pharmcy. Hacetlcpe University, Hacettepe, 06100 Ankara, Turkey

[2] Oravax, Inc., Department of Dental Research, Bidg. 2832 Ernie Pyle Road, Fort Meade. Maryland 20755.

[3] School of Pharmacy, Taipei Medical College. 250Wu-Hsing Street, Taipei. Taiwan.

[4] College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0082.

[5] To whom correspondence should be addressed.

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## Methods

### *Preparation and Characterization of Blank PLGA Microspheres*

Blank PLGA microspheres were prepared by an aqueous emulsion-solvent extraction/evaporation technique (17). In brief, a solution of PLGA in methylene chloride ($CH_2Cl_2$) was dispersed into a continuous phase consisting of 0.4% sodium oleate in water. The microspheres were solidified upon removal of $CH_2Cl_2$ at 40°C, rinsed with water and freeze dried. Seven batches of microspheres were prepared and mixed to provide sufficient quantity of homogenous microspheres. The microspheres were characterized for panicle size distribution, specific surface area and surface morphology by scanning electron microscopy.

### *Particle Size Distribution*

The size of the microspheres was determined after suspending the microspheres in 0.05% w/v Tween 80 solution using a Malvem Laser Diffraction Particle Sizer model 2600 (Southborough, MA).

### *Specific Surface Area*

The specific surface area of microspheres was determined by B.E.T. (Brunauer, Emmett and Teller) method using a Micromeritics model ASAP 2000 (Norcoss, GA). The B.E.T. method is based on the adsorption and desorption of nitrogen and krypton gas at the surface and within the pores of the microsphere sample.

### *Surface Morphology*

Surface Morphology of the microspheres was assessed by scanning electron microscopy (SEM) using a Hitachi Model S-800 Scanning Electron Microscope. A uniform layer of microspheres was fixed onto a double adhesive tape and coated with gold/palladium.

### *Analysis of sCT*

sCT was assayed by high performance liquid chromatography (HPLC) (Shimadzu, Columbia, MD) using a 250 x 4 mm Bio-Sil ODS-10 reversed phase column (Bio-Rad, Richmond, CA), eluted with a mobile phase consisting of A 0.1% trifluoroacetic acid in water and B: 0.1% TFA in acetonitrile (18). The gradient profile was 25% to 40% B in 8 minutes. The eluent was monitored for sCT at a wavelength of 220 nm. Calibration curve for the validated HPLC assay of sCT in 0.01 M phosphate buffer, pH 7.4, at a concentration range of 31.2 to 500 μ-g/ml had a correlation coefficient greater than 0.99. The retention time for sCT was 13.6 minutes. The coefficient of variation for three determinations was 5.33%. Samples were filtered through Millipore GV 0.22 u.m filters before 'mecrion.

### *Adsorption of sCT to Microspheres*

1.5 ml capacity polypropylene vials to which adsorption of sCT was found to be negligible were used in the adsorption studies. Various amounts of microspheres (2.5, 5.0, and 10 mg) were added to 1 ml of 1 mg/ml sCT solution in 0.01 M phosphate buffer, pH 7.4, placed on a rotary wheel and allowed to interact for specified periods of time at 25°C. Samples were centrifuged at 12,000 rpm and the supernatants were analyzed for sCT by HPLC.

In order to determine the extent of adsorption of sCT to the microspheres, an experiment with 5 mg microspheres was carried out for 6 days. Each day all of the supernatant was removed for analysis and replaced with fresh sCT solution at a concentration of 1 mg/ml. After the 6th day the microspheres were isolated for SEM.

### *Adsorption Kinetics and Isotherms*

Adsorption kinetics of sCT to the microspheres (10 mg) was evaluated using six sCT concentrations (50 μg/ml to 350 μg/ml in 0.01 M, pH 7.4, phosphate buffer). Residual sCT in the adsorption medium was periodically assayed by HPLC.

Based on the data obtained from the adsorption kinetics, equilibrium time was determined and adsorption isotherms were constructed utilizing the Langmuir (19) and Freundlich (20) models of adsorption.

*Langmuir Model*. The Langmuir equation is given by:

$$\frac{x}{m} = \frac{k_1 \,.\, k_2 \,.\, Ceq}{1 + k_1 \,.\, Ceq} \qquad (1)$$

where $x$ is the amount ofpeptide adsorbed, $m$ is the mass of microspheres, $Ceq$ is the concentration of unadsorbed peptide at equilibrium, $k_1$ is the affinity constant of the peptide for the microspheres and $k_2$ is the plateau surface concentration (μg/mg), usually defined as the monolayer concentration (2), a constant indicating the capacity of the microspheres for a given peptide. A plot of $Ceq$ versus $xlm$ should yield a rectangular hyperbola. Equation 1 can be rearranged as

$$\frac{Ceq}{x/m} = \frac{k_1 \,.\, k_2}{1} + \frac{Ceq}{k_2} \qquad (2)$$

A plot of $Ceq/(x/m)$ versus $Ceq$ should yield a slope of $1/k_2$ and an intercept of $1/(k_1k_2)$. The derivation of equation 2 is dependent upon several assumptions: The adsorbed layer is confined to a monolayer, the adsorbate solution is very dilute, all sites available for adsorption are energetically equivalent and there are no lateral interactions between adsorbate molecules.

*Freundlich Model*. The Freundlich equation is given by:

$$\frac{x}{m} = k \,.\, Ceq^{p} \qquad (3)$$

where $k$ is a constant related to the capacity of the adsorbent for the adsorbate, $p$ is a constant related to the affinity of the adsorbent for the adsorbate. Logarithmic transformation of the above equation gives:

$$log\left(\frac{x}{m}\right) = log\ k + p\ log\ Ceq \qquad (4)$$

A plot of $x/m$ against $Ceq$ on a log-log scale gives the values of $k$ and $p$. The Freundlich model predicts infinite adsorption at infinite concentration. The main assumptions are: the ad-

sorbed layer is not confined to a monolayer and lateral interactions between adsorbate molecules may be possible.

## RESULTS AND DISCUSSION

### Characterization of PLGA Microspheres

The particle size of each batch was measured individually and mean diameters of the microspheres were determined to be 14.8 ± 1.3 μm. The specific surface area of the mixed batch was 0.32 $m^2/g$. SEM revealed that the microspheres were spherical, non-porous and have a uniform distribution.

### Adsorption of sCT to Microspheres

The adsorption of sCT to microspheres at various microsphere concentrations is presented in Figure 1. The kinetic profiles can be divided into three regions, the pre-lag phase, the lag phase and the phase of rapid adsorption. In these three regions, different phenomena may be occurring. In the pre-lag phase adsorption of about 30-70 μg/mg sCT occurred, presumably forming a monolayer. In the lag phase an alteration of either the polymer surface or the peptide in bulk or the adsorbed peptide occurred which resulted in the phase of rapid peptide adsorption. The lag phase was longer with the 2.5 mg sample than the 5 mg sample, while there was no discernible lag phase with the 10 mg sample. The lag phase appears to be dependent on the polymer surface area. Following the lag phase, peptide adsorption was rapid and proceeded at a rate which appears to be independent of the polymer mass used for adsorption. The adsorption ceased only upon exhaustion of the peptide in solution. The sample with 10 mg microspheres reached maximum adsorption in 12 hours, compared to 24 hours for 5 mg and 48 hours for 2.5 mg concentration. More than 90% of the added sCT was depleted from the solutions at the end of 48 hours irrespective of the microsphere concentration. The samples with the lowest weight of microspheres and hence lowest surface area adsorbed the most sCT per unit or surface area and therefore had the maximum surface concentration of sCT. Thus, if more sCT were available, the samples with 5 and 10 mg microspheres would adsorb at least 100 μg/mg. To verify this argument another experiment was conducted where the microspheres were suspended in sCT solution which was completely replaced every 24 hours with fresh sCT solution. About 80% of the sCT present in the solution was removed in each 24 hour period. At the end of 6 days a total of 5.1 mg sCT was removed from the combined solutions by 5 mg microspheres. In molar concentrations, each mole of PLGA removed about 10 moles of sCT in 6 days. The extent of removal per 24 hour period correlates with our previous finding on adsorption of sCT to powdered polymers (15). Samples of microspheres with adsorbed sCT showed two distinct

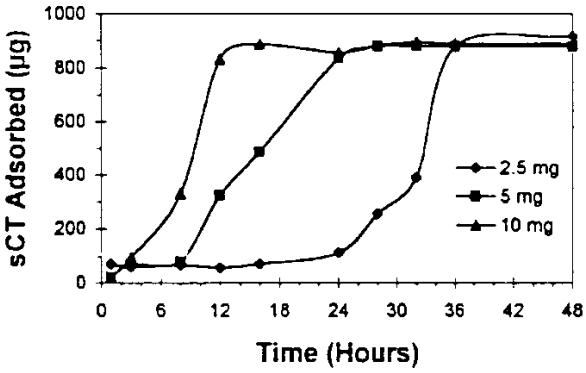


**Fig. 1.** Time course of sCT adsorption to PLGA at various microsphere concentrations. Microspheres were suspended in a 1 mg/ml sCT solution in 0.01 M phosphate buffer, pH 7.4.

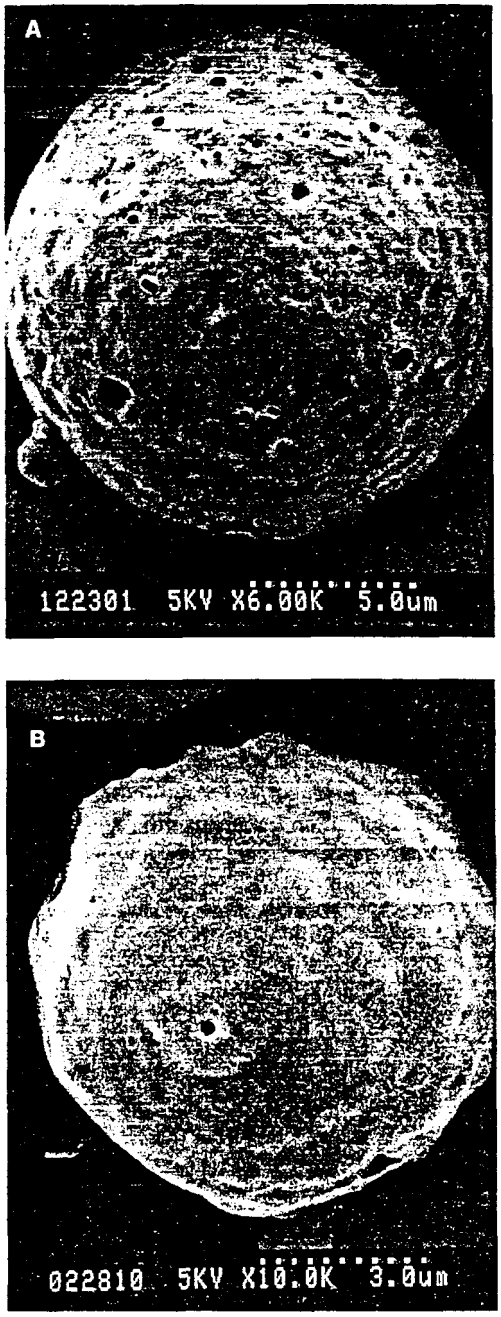


**Fig.** 2. Scanning electron micrographs of blank PLGA microspheres (A): Before adsorption, (B): Suspended in phosphate buffer, pH 7.4, containing sCT for 6 days.

sedimentation layers after centrifugation; the lower layer was opaque and dense while the upper layer was translucent and loosely packed. sCT solutions without microspheres undergoing the same treatment for 24 hours did not show any change in the sCT concentration by HPLC.

SEM photomicrographs of blank microspheres (Figure 2A) show a smooth surface, whereas microsphere suspended in peptide solution (Figure 2B) show a considerable amount of surface accumulation (presumably peptide) after 6 days. This indicates that the continuous depletion of sCT by PLGA microsphere surface appears to be a result of initial adsorption of the peptide to the polymer surface with subsequent interaction of the peptide in solution with the already adsorbed peptide. It is speculated that the integrity of the multilayer may be maintained by the hydrophobic interaction between peptide molecules (14).

**Adsorption Kinetics and Isotherms**

Figure 3 shows the removal of sCT from solution over a shorter time period using six sCT concentrations (0.05 mg/ml to 0.35 mg/ml) in 0.01 M, pH 7.4, phosphate buffer. At all concentrations there was an initial uptake of 10 to 30 μg sCT during the first 30 minutes. Adsorption continued after a short lag phase and an equilibrium was attained between 60 and 90 minutes. At lower concentrations, 50 and 100 μg/ml, the equilibrium appeared to be a true equilibrium, however, at higher concentrations, the equilibrium was transient and adsorption continued after the equilibrium. The time at transient equilibrium decreased with increasing concentration of sCT. The second phase of adsorption at higher sCT concentrations can be attributed to formation of multiple layers of peptides on the surface of the polymeric microspheres. It appears that after the transient equilibrium changes occur in the bulk solution and at the polymer surface. In the solution peptide-peptide interaction may be occurring while at the polymer surface there is peptide reorientation (unfolding). These processes may lead to further interaction at the surface. When this occurs there is no longer equilibrium but probably precipitation at the surface.

The odsorption which results in a transient equilibrium during 60 to 90 minutes was used to construct the adsorption isotherms. The amount of sCT adsorbed from three consecutive time points in the equilibrium region were averaged to obtain the data plotted. It appears that the adsorption followed two different models depending on the concentration of the peptide in solution (Figure 4a (Inset)). At lower concentrations, equilibrium was attained but at higher concentrations the adsorption continued beyond the monolayer formation. The first four concentration points in the isotherm were evaluated by the Langmuir equation using native and linearized models and the results are plotted in Figure 4b. The data show a good correlation to the Langmuir model with a correlation coefficient of 0.987 from the non-linear fit and 0.997 from the linear fit indicating formation of a monolayer. The affinity constant calculated using equations 1 and 2 from the best-fit curve was 0.090 and the plateau surface concentration was 5.1 μg/mg. The surface concentration falls within the range reported for other peptides and proteins for hydrophobic surfaces (21-23). Concentrations higher than 150 p.g/ml were treated using the Freundlich model as shown in Figure 4c. The data show correlation coefficient of 0.989 for a linear fit indicating multi-layer adsorption. The affinity constant calculated using equation 4 is 0.0246 and the capacity constant is 23.97 μg/mg.

In comparing the constants from Freundlich treatment of higher concentrations with those obtained from the Langmuir treatment of lower concentrations, it is evident that the

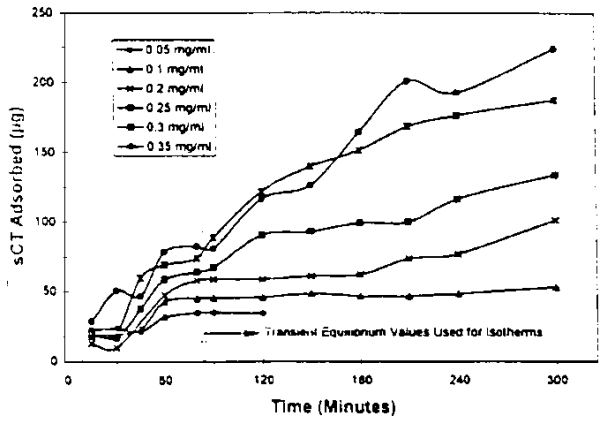


**Fig. 3.** Adsorption kinetics of sCT onto 10 mg PLGA microspheres. (sCT cone. range: 50-350 μg/mi in 0.1 M phosphate buffer, pH 7.4).

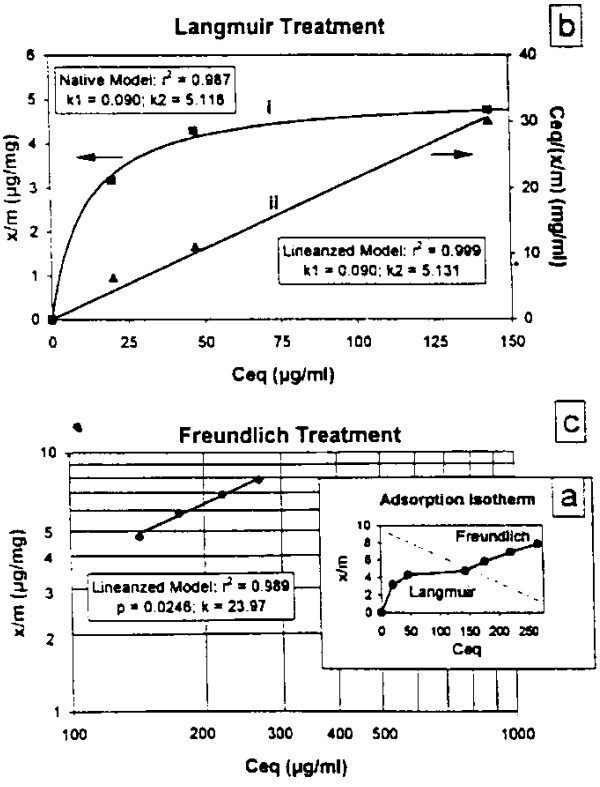


**Fig. 4.** (a) Adsorption Isotherm of sCT onto PLGA microspheres; (b) Langmuir treatment of the adsorption isotherm at sCT concentrations of 50 to 150 μg/ml (i): Native Model, (ii): Linearized Model; (c) Freundlich treatment of the adsorption isotherm at sCT concentrations of 150 to 350 u.g/ml.

adsorption at higher concentrations has lower affinity and higher capacity than that at lower concentrations. This may be due to the fact that peptide molecules are in monomeric form and the hydrophobic interaction between the peptide and the polymer is strong. At higher peptide concentrations the hydrophobic interaction between the peptide molecules may be stronger resulting in self-association of peptides. Adsorption may have occurred when self-association was complete. Then, the self-associated molecules adsorbed onto the polymeric surface forming multiple layers.

The adsorption isotherms suggest these predictions: In dilute solutions, peptide-polymer interaction favored monolayer adsorption which fitted the Langmuir model as all the adsorption sites on the polymer surface are expected to be energetically equivalent. At higher peptide concentrations peptide-peptide interactions are favored ultimately resulting in multilayer adsorption which fitted the Freundlich model.

The results suggest that adsorption occurred as a result of three phenomena: (1) peptide-polymer interaction—absorption of peptide onto the polymeric surface. (2) peptide-peptide interaction on the polymer surface, in which adsorption of peptide onto polymer was followed by adsorption onto previously adsorbed peptide layer; this resulted in formation of multilayer as observed in the continuous depletion of sCT from the adsorption medium. (3) Adsorption onto polymer at high peptide concentrations wherein self-association of peptide molecules (peptide-peptide interaction) preceded adsorption onto the polymer resulting in multilayer formation. This was evident from the delayed adsorption of sCT in the kinetic studies.

PLGA microspheres have a high adsorption capacity for sCT which is dependent on both peptide and polymer concentrations. Desorption of the absorbed sCT to polymer has been reported in detail elsewhere (14). Controlling the desorption over a favorable time should enhance the potential for applying the phenomenon of adsorption for controlled delivery of peptides.

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