Peptide Liquid Crystals: Inverse Correlation of Kinetic Formation and Thermodynamic Stability in Aqueous Solution

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INTRODUCTION

Luteinizing Hormone Releasing Hormone (LHRH) is a highly potent decapeptide that stimulates the release of luteinizing hormone and follicle stimulating hormone. Although it was initially thought that the LHRH agonists would be useful for the treatment of infertility, continuous administration of agonist resulted in a parodoxical desensitization of pituitary gonadotropes by receptor down regulation, causing suppression of fertility.[1] These suppressive effects, however, may be very useful for the treatment of other diseases, including endometriosis, breast cancer, precocious puberty and prostatic cancer.

The aqueous formulation of hydrophobic LHRH agonists and antagonists (such as deterelix, Figure 1) is challenging because of unwanted peptide aggregation manifested as peptide liquid crystals (PLC), turbidity, precipitation and gelation[2,3]. Although a few studies have been reported on PLC growth and stability in nonaqueous solvents[4], there is little information of PLCs in aqueous solution. Information of this nature would be useful for the aqueous formulation of hydrophobic peptides. In order to understand PLC growth and stability in aqueous solution, we measured the onset of PLC formation, as well as the PLC critical melting temperature (T_{cm}) of a model LHRH peptide. Herein we report the unusual effect of added buffer anions on the kinetics and thermodynamics of peptide PLCs in aqueous solution.

MATERIALS AND METHODS

Materials: Deterelix acetate was synthesized by the Institute of Organic Chemistry (Syntex Research). Water was purified by filtration and ion-exchange (Milli-Q). Reagents used for buffer preparation (acetate and phosphate) were purchased from J.T. Baker and used without further purification. The salts Na_2SO_4, NaBr, NaCl, NaF, NaOAc, and HCOONa were supplied by Mallinkrodt. Buffers were prepared by mixing solutions of conjugate base and acid at known concentrations until the desired pH was reached. A Sensorex pH combination electrode and a Fisher Accument Model 629 pH meter was used to measure pH.

Liquid Crystal Formation and T_{cm} Measurement: A Leitz optical microscope (Model II PO1-BK Ortholux) was used to measure to the formation of liquid crystals and critical melting temperatures (T_{cm})[5]. Peptide solutions of known composition were loaded into rectangular, glass capillary microslides (50 × 3 × 0.3 mm, Vitro Dynamics Inc. N.Y.) by capillary action. Slides were stored at 5 °C until ready for viewing, and then observation was made at room temperature. The onset of liquid crystal formation was determined by viewing the microslide under crossed polars at known time intervals, usually every few hours for the first day, and daily thereafter. T_{cm}'s were determined by heating the microslide containing liquid crystals at 3 °C/minute until all traces of liquid crystals were gone, as measured by the disappearance of optical birefringence under crossed polarized light. Typically, the isotropic melting transition (from birefringent liquid crystals of undulose extinction, to black) was 2–4°C.

RESULTS AND DISCUSSION

Formulation of deterelix, a hydrophobic LHRH peptide, in aqueous solution resulted in the rapid formation of nematic PLCs of undulose extinction, with a birefringence less than 0.001 as determined using the Michel Levy chart[6]. An example of the PLC habits is shown in Figure 2. The onset of PLC formation was dependent on the concentration of deterelix. For example, deterelix at 1 mg/mL did not form PLCs, whereas the onset of PLCs occurred rapidly at 10 mg/mL (1 week) or 20 mg/mL (1 day). PLC formation is concentration-dependent because a critical volume fraction of compound, ϕ', must be reached before PLC formation can occur[7]. In this study, once PLC formation occurred as determined by the onset of optical birefringence, the extent of birefringence did not increase significantly over time. This leveling-off of birefringence with increasing ϕ above ϕ^*, the volume fraction where the medium is completely anisotropic, has been seen previously in other systems[3,7]. In our hands, most of the solutions formed PLCs before macroscopic gelation was observed (providing $\phi > \phi'$), indicating that PLC formation may be a useful early indicator for peptide aggregation.

When electrolytes were added to the aqueous peptide formulation (essentially a charged colloidal, macromolecular system) the solution properties were dramatically affected. The addition of electrolyte reduces electrostatic repulsion between the positively-charged molecules, with a concomitant increase in the aggregation number and decrease in the critical micelle concentration (cmc)[8]. In general, the effect of electrolytes on T_{cm} agreed with the established empirical

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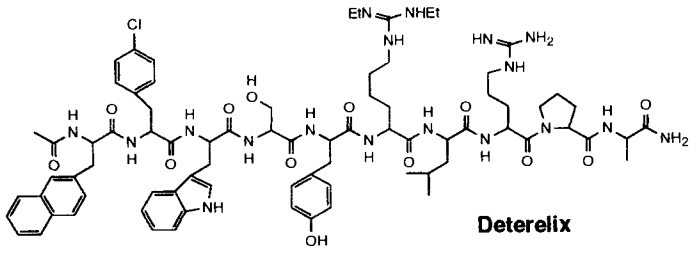

Figure 1. Structure of deterelix.

rules for peptide and protein aggregation by hydrophobic salts. In the Hoffmeister series of anion hydrophobicity, sulfate and other dianions are the most hydrophobic, followed by anions of moderate hydrophobicity such as bromide and chloride, and then hydrophilic counterions such as acetate, formate and fluoride[9]. The results of Table I show that PLC stability with added counterions paralleled counterion hydrophobicity: $SO_4^{=} > Br^- > Cl^- > AcO^- > HCO_2^- > F^-$. This effect of electrolyte is caused by the differential interaction of counterions with water in the bulk phase and in the PLC phase of lower polarity. Counterion interaction with water is highly dependent on ion size, where small, highly charged ions (such as fluoride) interact more strongly with bulk water molecules than do large polarizable ions (such as bromide) which partition more favorably into the PLC, thus effectively neutralizing peptide charge and resulting in tighter peptide packing[10]. Divalent ions such as $SO_4^{=}$ are also effective at stabilizing PLCs because a single dianion neutralizes the charge of multiple cations, allowing for tighter packing, increased aggregation number, and thus more stable PLCs. PLCs of deterelix in $SO_4^{=}$ solutions were so stable that they did not reach T_{cm}, even when heated up to >95°C. We also found that phosphate and dicarboxylate salts such as oxalate, succinate and adipate promoted PLC stability and high T_{cm}s (data not shown).

In contrast, the onset kinetics of PLC formation with added electrolytes showed results opposite of what was expected. Although the addition of any sodium salt increased the rate of PLC formation (as predicted), the rate was fastest with hydrophilic anions (such as acetate) than with hydrophobic ions (such as bromide or sulfate). This is surprising because PLCs with hydrophobic salts were thermodynamically more stable (as shown by their higher T_{cm}s, Table II) and might *a priori* be expected to form faster. This may be due to differential desolvation and/or nucleation effects. For example, the formation of stable PLCs may require extensive peptide or counterion desolvation, making the onset of PLC formation slow, but once the aggregates form they are not easily resolvated, and thus are stabilized. The onset of PLC formation is also determined by aggregate nucleation. According to colloidal theory, the major determinant for the onset of nucleation of protein crystallization is the balance between Van der Waals and electrostatic forces[11]. An increase in electrolyte content decreases intermolecular electrostatic repulsion forces, and thus promoting aggregate and PLC formation (as observed)[12]. The physical basis for the opposing kinetic and thermodynamic effects with added electrolyte is speculative, but it is clear from these and earlier studies[3] that the thermodynamic stability of PLCs is a misleading indicator of the time required for their formation.

These data show that peptide aggregation in aqueous solution may be exacerbated by the addition of both hydrophilic and hydrophobic counterions. In general, the prevention of PLCs by added buffer counterions is difficult to predict because of opposing kinetic and thermodynamic forces for salts of different hydrophobicity. Based on these observations, it is likely that other excipients (such as organic

Table I. Effect of Added Counterions on the Thermodynamic Stability of Deterelix as Measured by T_{cm} (°C).

Added Salt (M)[a]	$SO_4^{=}$	Br^-	Cl^-	HCO_2^-	AcO^-	F^-
0[b]	52	52	52	52	52	52
0.001	64	71	49	54	54	54
0.01	91	77	50	54	54	55
0.1	>95	>92	85	73	73	66

[a] Experimental conditions: 10 mg/mL deterelix at 5 °C with added sodium salts.

[b] In each experiment there was ~0.007 M acetate from 10 mg/mL deterelix acetate used in preparing the solutions. In the absence of added salt, deterelix at 10 mg/mL formed liquid crystals in ~7 days.

Table II. Effect of Added Counterions on the Time Required (days) for Deterelix PLC Formation.

Added Salt (M)[a]	$SO_4^{=}$	Br^-	Cl^-	HCO_2^-	AcO^-	F^-
0[b]	7	7	7	7	7	7
0.01	1	0.1	0.1	1	0.1	0.1
0.03	5	1	0.1	0.1	0.1	0.1
0.05	5	1	0.1	0.1	0.1	0.1
0.08	6	4	1	1	0.1	0.1
0.1	ppte	4	1.5	1	0.1	1

[a] Experimental conditions: 10 mg/mL deterelix at 5°C with added sodium salts (final pH 5–6).

[b] In each experiment there was ~0.007 M acetate from 10 mg/mL deterelix acetate used in preparing the solutions. In the absence of added salt, deterelix at 10 mg/mL formed liquid crystals in ~7 days.

Figure 2. Effect of NaCl on liquid crystal formation of 4 mg/mL deterelix after 3 weeks at room temperature. These micrographs were made under crossed polarized light. The left part of the slide is air only (no formulation buffer) and thus is dark. The curved meniscus separates the air phase from the formulation phase containing birefringent liquid crystals (shown as intense white). Various NaCl concentrations were used: top, 1.5 mM; middle, 7.5 mM; bottom, 15.4 mM.

cosolvents[3]) will be required to prevent PLC formation in aqueous parenteral peptide formulations.

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