Abstract: Understanding the secondary structure of peptides is important in protein folding, enzyme function, and peptide-based drug design. Previous studies of synthetic Ala-based peptides (>12 a.a.) have demonstrated the role for charged side chain interactions involving Glu/Lys or Glu/Arg spaced three \((i, i + 3)\) or four \((i, i + 4)\) residues apart. The secondary structure of short peptides (<9 a.a.), however, has not been investigated. In this study, the effect of repetitive Glu/Lys or Glu/Arg side chain interactions, giving rise to E-R/K helices, on the helicity of short peptides was examined using circular dichroism. Short E-R/K–based peptides show significant helix content. Peptides containing one or more E-R interactions display greater helicity than those with similar E-K interactions. Significant helicity is achieved in Arg-based E-R/K peptides eight, six, and five amino acids long. In these short peptides, each additional \(i + 3\) and \(i + 4\) salt bridge has substantial contribution to fractional helix content. The E-R/K peptides exhibit a strongly linear melt curve indicative of noncooperative folding. The significant helicity of these short peptides with predictable dependence on number, position, and type of side chain interactions makes them an important consideration in peptide design.

Keywords: \(\alpha\)-helix; E-R/K peptides; peptide design; salt bridges; circular dichroism

Introduction
Understanding and modifying secondary structure in peptides is necessary in understanding protein folding,\(^1\) in engineering proteins,\(^2\) and in designing enzymatic targets.\(^3\) Since the discovery of significant helicity in the 13 amino acid C-peptide from ribonuclease A,\(^4,5\) there has been a considerable amount of research on helicity of \textit{de novo} peptides. Peptides ranging from 12 to 30 residues have been studied to understand the interplay between side chain interactions, the helix dipole, and the intrinsic helix-forming ability of each amino acid.\(^6\) This research has revealed that Ala-based peptides show substantial helix formation, as Ala has a high helix propensity.\(^7\)

Ala-based peptides have been used to look at side chain interactions, such as Glu with Lys (E/K) salt bridges which form stabilizing \(i \rightarrow i + 3\) and \(i \rightarrow i + 4\) interactions.\(^8\) In Ala-based peptides containing \(i \rightarrow i + 4\) E/K salt bridges \((\text{EAAAK})_n\), replacing Lys with Arg was found to enhance the helical content of the peptides.\(^9\) Studies were also done to examine peptides \((\geq 16\) amino acids) that have repetitive Glu-Lys and/or Glu-Arg interactions (which we refer to as the E-R/K motif). For example, Lyu et al.\(^10\) examine one 18-residue peptide consisting of a repeating motif of four negatively charged Glu residues followed by four positively charged Lys residues. This motif of four Glu residues followed by a...
combination of four Lys and/or Arg residues has been found in a variety of proteins, including caldesmon,\textsuperscript{11} myosin X, and myosin VI.\textsuperscript{12,13} It has been shown to form long (up to 30 nm), single, stable, and relatively rigid helices (persistence length = 15 nm) in various proteins.\textsuperscript{14–16}

Experimental measurement of helicity using circular dichroism of peptides has been used in conjunction with statistical mechanics models of helix–coil transition\textsuperscript{17,18} to develop prediction programs for the helicity of peptides. The original Zimm and Bragg\textsuperscript{20} and Lifson and Roig\textsuperscript{26} models described the helix–coil transition as a two-step process with helix nucleation followed by helix propagation or elongation. These theories were modified in subsequent studies to include side chain–side chain interactions\textsuperscript{10} and peptide-capping effects.\textsuperscript{21} Currently, one of the models, AGADIR, has been benchmarked against the largest selection of peptide sequences.\textsuperscript{22} AGADIR is an algorithm that is based on helix–coil theory but modified to incorporate experimentally derived parameters.\textsuperscript{18} The algorithm attempts to obtain an energetic description of the system by splitting the conformational energy of the peptide into a sum of energies: intrinsic helical tendencies of each residue, main chain–main chain hydrogen bonding, side chain–side chain interactions, helical dipole effects, and effects of nonhelical residues.\textsuperscript{23}

While AGADIR predicts the helicity for peptides greater than one helical turn or four residues, there is a lack of experimental data for the helicity of short peptides (<9 amino acids). Ala-based peptides demonstrate only marginal helicity when 10 residues in length.\textsuperscript{9} As a result of the focus of all helix prediction algorithms on Ala-based peptides, short peptides have largely been overlooked both in experimental and theoretical treatments.

This study shows that peptides shorter than 10 residues can in fact have significant helicity, even without helix-inducing solvents like trifluoroethanol. Short E-R/K–based peptides were studied using circular dichroism. These short peptides exhibited high helicity, and E-R–based E-R/K peptides showed significantly greater helicity than their E-K counterparts.

Results and Discussion

Short peptides demonstrate significant helicity
Peptides with four Glu residues followed by four Lys residues [the (E4K4)\textsubscript{n} motif] were studied, where \( n = 1, 2, \) or 3 [Fig. 1(a)]. All peptides in this study were designed with an N-terminal acetyl group and C-terminal amide cap. A Tyr was placed at the N-terminus separated by a Ser from the rest of the peptide to facilitate concentration measurements.\textsuperscript{23} Although the peptide bonds contributed by the Tyr and Ser residues are included in all calculations, we will refer to a peptide’s length by the number of helix-promoting residues (i.e., excluding Tyr and Ser). Not only were the 16-residue (E4K4)\textsubscript{2} and 24-residue (E4K4)\textsubscript{3} peptides helical as previously reported\textsuperscript{10} but also the 8-residue E4K4 peptide demonstrated significant helix content (59%). For the reference helix content of short peptides in the absence of salt bridges, Ala-based peptides were designed with either Arg or Lys for solubility purposes (A1–A3, Table I).

All three peptides have minima at 208 and 222 nm characteristic of \( \alpha\)-helices. The isodichroic point at 202 nm is also consistent with a system that occupies two different states, the structured helical state and the unstructured state.\textsuperscript{8,24} As the helix content of the peptide increases, the 208 nm minimum, which contains contributions from both the helical and the unstructured state, decreases. The helix content is most easily monitored by examining the mean residue ellipticity (MRE) at the 222 nm minimum. To compare our results with AGADIR predictions, the helix content was calculated in accordance with the Chen equation\textsuperscript{25} used by AGADIR.\textsuperscript{22} The 1974 Chen equation for calculating percent helix values was later tested with Ala-based peptides of varying chain lengths and was refined slightly.\textsuperscript{26} In the case of the E4K4 peptide, the helix content is high at 59%, close to the value predicted by the AGADIR algorithm (Table I).

**E-R peptides have higher helix content than E-K peptides**
To examine the effect of Arg versus Lys in these E-R/K peptides, the E4K4 peptide was initially compared with the E4R4 peptide [Fig. 1(b)]. Not only is the 222 nm minimum at a lower MRE but also the 208 nm minimum has less contribution from the unstructured conformation. From the 222 nm values, the helix content of the E4R4 peptide was calculated to be \( \sim 71\% \) versus 59% for the E4K4 peptide, again fitting well the prediction from the AGADIR algorithm (Table I). The thermal melts of these eight-residue long peptides were noncooperative and melting occurred over a wide temperature range (80°C), as is a characteristic of E-R/K peptides [Fig. 1(e)]. The melts were reversible (data not shown).

As high helicity was observed in eight-residue peptides, we examined even shorter peptides for helix content. Peptides six and five residues in length were examined [Fig. 1(c,d), respectively]. It must be noted that for peptides the unstructured state is a mixture of different backbone conformations, with contributions from polyproline II.\textsuperscript{24} The polyproline II spectrum is positive between 220 and 230 nm, which is consistent with positive CD values at 222 nm for a few of our peptides, such as A2 and A3.

The helix content for these six and five-residue peptides, listed in Table I, provide two important insights. First, peptides with this E-R/K motif exhibit surprisingly high helix content with only six or even five residues (~48% for E3R3 and ~19% for
Each additional $i \rightarrow i + 4$ and $i \rightarrow i + 3$ salt bridge increases the helix content of short peptides. In the case of E4R2 and E3R3, the E3R3 peptide, with an additional $i \rightarrow i + 3$ salt bridge, has a helix content nearly twice that of E4R2 (Table I). It is worth noting that this increase in helix content likely results from losing a potentially destabilizing $i \rightarrow i + 4$ electrostatic repulsion between the first and fourth E. According to AGADIR, losing this destabilizing interaction, although, only accounts for part ($\sim 60\%$) of the increase in helix content.

Second, in all cases, the E-R/K peptides that contain Arg have significantly higher helix contents than the corresponding peptides with Lys. For example, E3R3 has $\sim 48\%$ helix versus $\sim 15\%$ for E3K3. This trend has been previously reported in the context of Ala-based peptides. Knight et al. have hypothesized that E-R/K peptides would exhibit greater stability with E-R interactions relative to E-K interactions. The presence of the guanidinium group may enable Arg to interact simultaneously with the negative Glu residues in both directions.

Conclusions
Secondary structure of short peptides has largely been overlooked because of the general assumption...
that they are unstructured in solution. This study reports appreciable secondary structure of short peptides that are based on the E-R/K motif. These short peptides are stabilized by $i \to i + 4$ and $i \to i + 3$ salt bridges, with greater helix content in E-R–based peptides relative to E-K peptides. Further studies are needed to understand why E-R–based peptides have higher helix content. The existing prediction program AGADIR, however, did capture this difference between Arg and Lys and compared reasonably with experimental measurements. Overall, this study highlights the importance of the E-R/K motif in determining the secondary structure of short helices, and in de novo design of peptides. In the context of longer protein-derived E-R/K single $\alpha$-helices, it suggests that E-R–enriched helices could be more stable than their E-K counterparts. The structural and functional consequences of the relative abundance of R versus K residues in E-R/K helices remain to be determined.

### Materials and Methods

**Circular dichroism spectroscopy**

Peptides were purchased from GenScript and purified to $\geq 98\%$, as determined by mass spectrometry and HPLC (GenScript Corp, Piscataway, NJ). Peptide concentrations were calculated using the $e_{280} = 1490 \text{ M}^{-1} \text{ cm}^{-1}$. CD spectra were acquired using an Aviv 62DS instrument (Aviv Biomedical, Lakewood NJ) with a 1-mm path-length cell. Measurements were taken every 1 nm at 0°C with a 10 s averaging time and with concentrations ranging from 60 to 110 $\mu$M in 10 mM sodium phosphate buffer, pH 7.0. MRE was estimated from the following equation, 

$$\% \text{ helix} = 100 \left( \frac{[\theta]_{222} - 39500(1 - 2.57/n)}{39500} \right),$$

where $n$ is the number of total peptide bonds and $[\theta]_{222}$ has been corrected for background. Helix content values were compared with those predicted by the algorithm AGADIR.

### References

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