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Sequence length dependence in arginine/phenylalanine oligopeptides: Implications for self-assembly and cytotoxicity

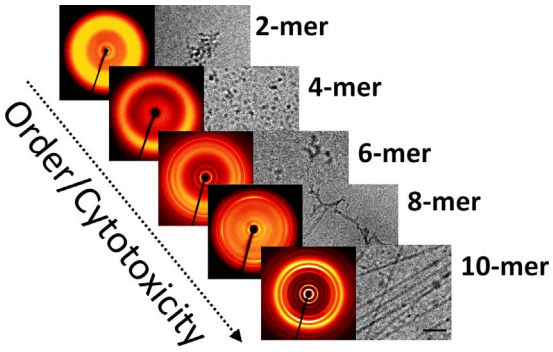
Emerson R. Silva[a,*], Eduardo Listik[b], Sang W. Han[a], Wendel A. Alves[c], Bruna M. Soares[c], Mehedi Reza[d], Janne Ruokolainen[d], Ian W. Hamley[e]

[a] Departamento de Biofísica, Universidade Federal de São Paulo, São Paulo 04023-062, Brazil
[b] Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo 05508-900, Brazil
[c] Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo André 09210-580, Brazil
[d] Department of Applied Physics, Aalto University, Aalto FI-00076, Finland
[e] Department of Chemistry, University of Reading, Reading RG6 6AD, United Kingdom

HIGHLIGHTS

- Progressive crystalline order is found upon sequence length in $[RF]_n$ peptides.
- Amyloid-β features are found in 8-mer and 10-mer sequences.
- Order correlate with cell death, suggesting that structure matters for cytotoxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

We present a detailed study on the self-assembly and cytotoxicity of arginine-rich fragments with general form $[RF]_n$ (n = 1–5). These highly simplified sequences, containing only two L-amino acids, provide suitable models for exploring both structure and cytotoxicity features of arginine-based oligopeptides. The organization of the sequences is revealed over a range of length scales, from the nanometer range down to the level of molecular packing, and their cytotoxicity toward C6 rat glioma and RAW264.7 macrophage cell lines is investigated. We found that the polymorphism is dependent on peptide length, with a progressive increase in crystalline ordering upon increasing the number of [RF] pairs along the backbone. A dependence on length was also found for other observables, including critical aggregation concentrations, formation of chiral assemblies and half maximum inhibitory concentrations (IC_{50}). Whereas shorter peptides self-assemble into fractal-like aggregates, clear fibrillogenic capabilities are identified for longer sequences with octameric and decameric chains exhibiting crystalline phases organized into cross-β structures. Cell viability assays revealed dose-dependent cytotoxicity profiles with very similar behavior for both glioma and macrophage cell lines, which has been interpreted as evidence for a nonspecific mechanism involved in toxicity. We propose that structural organization of $[RF]_n$ peptides plays a paramount role regarding toxicity due to strong increase of local charge density induced by self-assemblies rich in cationic groups when interacting with cell membranes.

* Corresponding author at: Department of Biophysics, Federal University of Sao Paulo, Rua Botucatu n° 862, 7° andar, CEP: 04023-062, São Paulo, Brazil.
E-mail address: er.silva@unifesp.br (E.R. Silva).

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1. Introduction

Self-assembly of oligopeptides into well-ordered nanostructures is a current topic due both to the outstanding properties of these compounds for developing new materials [1] and the involvement of peptide aggregates in several degenerative diseases [2,3]. Designed drug carriers [4], nucleic acid transporters [5] and biosensors [6] are among the main applications envisaged for peptide-based scaffolds [7]. In the field of clinical disorders, amyloid-related pathologies such as Alzheimer's and Parkinson's diseases are well-known examples of the role played by protein/peptide self-assembly in biological processes [8]. The polymorphism exhibited by these assemblies is complex and appears correlated to a number of factors, ranging from composition to synthesis conditions.[1] Also, the influence on living matter, especially concerning toxicity, is closely correlated to both chemical composition and structural organization [9]. Assessing the effects of these multiple parameters on final characteristics of the self-assemblies is a major challenge for the scientific community; however, despite this complexity, remarkable similarities and patterns are observed under certain conditions. For instance, amyloid aggregates originating from very different misfolding proteins possess a common structure typified by β-sheet-rich oligomers that assemble into long fibers organized according to a cross-β structure of paired β-strands running perpendicular to the long axis [2,10].

Interaction of cell membranes with β-sheet nanofibers is a topic research field due to the high potential of peptide-drug amphiphiles for intracell delivery and appearance of these structures in a variety of biological processes such as cell adhesion, signaling and cytotoxicity [11–18]. Newcomb et al. [11] investigated cytotoxicity onto surfaces coated with nanofibers obtained from two kinds of surfactant-like peptides; namely, $C_{16}H_{31}O$-$A_3G_3K_3$ and $C_{16}H_{31}O$-$V_3A_3K_3$ peptide amphiphiles (PAs). These PAs are characterized by cationic head groups (lysines) separated from alkyl tails by amino acid spacers able to drive the formation of β-sheet in the resulting nanofibers. Interestingly, they found that species containing valines as spacers form stable β-sheet-rich fibers, which are well-tolerated by cells. On the other hand, $C_{16}H_{31}O$-$A_3G_3K_3$ assemblies do not show β-sheet features and instead exhibit higher cytotoxicity levels. The reasons for this are related to higher intermolecular cohesion observed in β-sheets, which hinders rapid incorporation of peptide moieties from fibers into cell membranes, preventing disruption. When they removed one lysine residue from the valine-free PA, cytotoxicity was found to significantly decrease. This behavior, with decreasing cationic charge leading to lower cytotoxicity levels, is also consistent with other reports [19] which investigated cytoxicity in lipids and polymer scaffolds and highlights the role of charges in cytotoxicity. Recently, we have taken advantage of π-π stacking interactions and directionality provided by aromatic rings in phenylalanine side-chains and demonstrated the fibrillization behavior of a sequence composed only of alternating arginine and phenylalanine residues, [20] in the octamer $[RF]_4$. This sequence has been shown to comprise all main features of much more complex amyloid peptides, including their cross-β organization. It is notable that this feature has been unambiguously determined for a cationic sequence, abundant in arginine, which is not typical of native amyloidogenic sequences. L-Arginine is an amino acid widely found in cell-penetrating peptides which plays a central role in several biological processes [21]. Moreover, arginine-rich domains are often found in protein interaction hotspots [22] and appear at interfaces of liquid-liquid phase separation [23]. Thus, arginine-rich oligopeptides have particular behavior regarding interactions with other biomolecules, including cell membranes, and a proper understanding of structure-toxicity relationships in these species is well worth the effort. The current work is a step forward in this direction.

Herein, we investigate in detail both the structure and toxicity of alternating arginine/phenylalanine fragments with general form $[RF]_n$ (n = 1–5). The chemical structures of the peptides are shown in the Supporting Information. These highly simplified sequences, containing only two L-amino acids in their composition, provide suitable models for exploring both structural and toxicity features of arginine-based oligopeptides. The structure of these peptides is unveiled in detail, from the nanoscale down to the level of molecular packing, and their cytotoxicity toward C6 rat glioma and RAW264.7 macrophage cell models is investigated. We find a rich polymorphism with a close relationship between structure and length of the peptide chain. Critical aggregation concentrations detected through pyrene fluorescence assays exhibit a general trend to decrease upon increase in the number of [RF] repeats along the peptide backbone, possibly a consequence of diminution of charge density as the sequences become longer. The formation of irregular, oligomer-like, aggregates is observed even for the minimal [RF] dimeric species; however, fibrillization requires at least four phenylalanine groups in the sequence. Interestingly, whereas octameric sequences form cross-β structures with intertwined fibrils, the decamer $[RF]_5$ self-assembles into paired β-strands within single pleated β-sheets which twist into thicker helical fibrils. Toxicity assays show that IC50 values are also dependent on peptide length, with longer sequences exhibiting higher inhibitory rates. The viability profiles are quite similar between different cell lines, suggesting that cell death is caused by a nonspecific mechanism.

2. Materials and methods

Sample preparation: Alternating RF peptides (R = arginine, F = phenylalanine) were custom-synthesized by Peptide Protein Research Ltd. (Fareham, UK) and used as received. Four sequences were purchased from PPR Ltd.: $[RF]_1$, $[RF]_2$, $[RF]_3$ and $[RF]_5$. All peptides used trifluoroacetic acid (TFA) as a counterion and the purity of purchased peptides was determined by the manufacturer through HPLC and electrospray-mass spectroscopy immediately after synthesis, revealing the following purity levels: $[RF]_1$: purity = 97.6%, Mw = 322.2 g/mol (calculated: 321.4 g/mol); $[RF]_2$: purity = 98.7%, Mw = 625.5 g/mol (calculated: 624.7 g/mol); $[RF]_3$: purity = 98.6%, Mw = 927.7 g/mol (calculated: 928.1 g/mol); and $[RF]_5$: purity = 95.1%, Mw = 1535.2 g/mol (calculated: 1534.8 g/mol). $[RF]_4$ peptides were synthesized in our lab using standard solid-phase methods as described elsewhere [11,15]; however, herein, acetylation steps were not performed. This sequence was characterized in our lab, showing purity = 96.4% and Mw = 1231.6 g/mol (calculated: 1231.5 g/mol). Structural and cytotoxicity assays were carried out within 6 months after receiving (or synthesizing) the sequences. Additional characterization was made on the same peptide batches after ~24 months (dry powder storage at −20 °C) to assess stability and investigate elution times of all peptides under the same HPLC conditions. In despite some degree of degradation was noticed after this time, the batches kept purities > 90% for all $[RF]_n$ sequences and retention times were found to progressively increase upon length of $[RF]_n$ oligopeptide (see Fig. S3). HPLC and mass-spectroscopy data from both "fresh" and "aged" batches may be found in the SI file. Samples used in physico-chemical assays were prepared by dissolving the appropriate quantity of peptide into milli-Q water (or D_2O for SANS and FTIR assays) at the desired final concentration. To ensure the formation of self-assemblies, samples were left to rest for at least 24 h prior to further characterization. In the case of fluorescence assays, solutions were kept at room temperature for 1 day prior to analysis. Prior to SAXS, SANS and cryo-TEM experiments, samples were kept in the fridge at 4 °C for a period of ~1 week. For fiber XRD, aged solutions (~4 weeks) were used for preparing the stalks. **Fluorescence assays**: A Carian Varian Ellipse spectrometer was used to obtain fluorescence data. Formulations containing peptides at concentrations ranging from ~1 μM to ~30 mM were prepared using a 5 μM pyrene solution and the corresponding emission spectra were recorded upon illumination with an excitation wavelength λ_{exc} = 338 nm. Samples were transferred into 10 mm width quartz cuvettes and emission spectra were recorded in the

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