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Modification of gelation kinetics in bioactive peptide amphiphiles

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ABSTRACT

Peptide amphiphiles (PAs) previously designed in our laboratory are known to self-assemble into nanofibers that exhibit bioactivity both *in vitro* and *in vivo*. Self-assembly can be triggered by charge neutralization or salt-mediated screening of charged residues in their peptide sequences, and the resulting nanofibers can form macroscopic gels at concentrations as low as 0.5% by weight. Controlling the kinetics of gelation while retaining the bioactivity of nanofibers could be critical in tailoring these materials for specific clinical applications. We report here on a series of PAs with different rates of gelation resulting from changes in their peptide sequence without changing the bioactive segment. The pre-existence of hydrogen-bonded aggregates in the solution state of more hydrophobic PAs appears to accelerate gelation kinetics. Mutation of the peptide sequence to include more hydrophilic and bulky amino acids suppresses formation of these nuclei and effectively slows down gelation through self-assembly of the nanofiber network. The ability to modify gelation kinetics in self-assembling systems without disrupting bioactivity could be important for injectable therapies in regenerative medicine.

1. Introduction

Self-assembly is an attractive strategy to introduce biomaterials into living tissues due to the versatility it offers for delivery methods and its potential for structural control at the nanoscale. Self-assembling systems could be used in minimally invasive therapies as biomaterials that can be injected as fluids which solidify once inside the body by physiological triggers such as heat or salinity [1–5]. Depending on the application, the desired time to gelation could vary from a time scale of seconds up to several minutes. While shorter gelation times allow for more targeted delivery of the therapeutic, longer gelation times can be desirable in the case where a gelating material must be delivered to an area of the body with little void space and high tortuosity, such as the spinal cord. Too rapid gelation in such applications can lead to clogging of the delivery needle and failure of the material to diffuse throughout the affected area. Such issues of delivery motivated the current work, which attempts to describe the factors that influence gelation speed of nanofiber-forming peptide amphiphiles (PAs).

We previously reported on a class of self-assembling PAs consisting of a lipophilic segment attached to the N- or C-terminus of a short peptide with one or more charged residues, that self-assemble to form nanofibers of 5–8 nm in diameter and up to microns long [5–11]. The lipophilic segment of the PA forms the core of the nanofiber and the peptide portion is nearest to the fiber surface. Electrostatic charges in the structure of PAs and the consequent intermolecular repulsion among molecules prevent long nanofibers from forming spontaneously in solution. If this charge is neutralized or shielded, for instance by the addition of electrolytes or by adjustment of pH, fiber formation is energetically favored by hydrophobic sequestration of the alkyl tails away from water and the potential for hydrogen bonding between the peptide segments. PAs in this class characteristically form assemblies rich in β-sheet-type hydrogen bonding [8,12–14]. These β-sheets are believed to play a role in determining the cylindrical nanofiber morphology, which is robust to major perturbations of the peptide sequence. According to classical models for surfactant self-assembly, as the hydrophilic headgroup becomes bulkier relative to the tail the cylindrical morphology should give way to spherical micelles [15]. This transition does not seem to happen easily in these PAs, presumably due to the large energetic reward for hydrogen bonding in β-sheets [8,16]. The nanofibers formed by our PA designs contain β-sheets that tend to align parallel to the long axis of the nanofibers to an extent which is sequence dependent

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and have as well some degree of order in their hydrophobic alkyl cores [17]. This robustness enables the incorporation of large varieties of bioactive peptide sequences into the PAs without disrupting fiber formation. Possible bioactive peptides include the extensively studied cell adhesion sequence RGD from fibronectin [18] and the laminin-1 sequence IKVAV [19,20] known to promote neurite outgrowth. PAs containing RGD [4,6,9,11,21,22] and IKVAV [5,8,11,13,23] have been studied and found to be promising biomaterials. In particular an IKVAV-bearing PA has been shown to promote neurite outgrowth and selective and rapid differentiation of mouse neural progenitor cells into neurons [5], a result that has important implications in the use of these biomaterials in spinal cord injury repair.

It has been established that the final mechanical properties of PA nanofiber gels can be tuned by modulating interactions among the self-assembling monomers [9,12,24]. Previous work in our laboratory demonstrated that PA mechanical properties can be tuned by adjusting the strength of intermolecular ionic bonding, which enables different mechanical regimes to be accessed without modifying the molecule [9,25]. Paramonov et al. have also shown that it is possible to tune PA mechanical properties by systematic mutation of N-methylation of glycines [12] or by the inclusion of phospholipids in the assembled structure [24], both of which serve to disrupt the hydrogen bonding that is critical for fiber formation and lower the mechanical strength.

In order to maximally exploit the advantages of self-assembly, it is desirable to control not only matrix structure and function but also the kinetics of assembly, which to our knowledge has not been systematically studied for this type of molecule. The kinetics of gelation would be particularly important for biomaterials that are designed for minimally invasive therapies to be injected as liquids and form gels *in situ*. In this paper we report on three series of epitope-bearing PAs in which the rates of gelation under identical conditions have been varied through modifications in peptide sequence. We also investigate in one of the systems whether these changes in kinetics affect bioactivity. These results could have significant impact on the design of future generations of PAs.

2. Materials and methods

2.1. Materials

Resin for solid phase synthesis and some amino acids were purchased from EMD Biosciences (La Jolla, CA). The remaining amino acids and synthesis reagents were purchased from Sigma–Aldrich (St. Louis, MO) and Anaspec, Incorporated (San Jose, CA). Solvents were purchased from VWR International (West Chester, PA) and Sigma–Aldrich.

2.2. PA synthesis

The PAs were prepared as described previously [9,12] using standard fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 433A automated peptide synthesizer. All peptides prepared have a C-terminal carboxylic acid and were made by using prederivatized Wang resins. After the peptide portion of the molecule was synthesized, the resin was removed from the automated synthesizer and the N-terminus was capped with palmitic acid. Cleavage and deprotection of the PAs were done with a mixture of triflouroacetic acid, water, and triisopropylsilane in a ratio of 95:2.5:2.5 for 2–3 h at room temperature depending on the sequence. The cleavage mixture and two subsequent dichloromethane washings were filtered into a round-bottom flask. The solution was concentrated *in vacuo* to a thick viscous solution by rotary evaporation, then triturated with cold diethyl ether. The white precipitate was collected by filtration, washed with copious cold ether, and dried overnight. All PAs were purified on an XBridge® C18 reverse phase column (Waters Corporation, Milford, MA) using high pressure liquid chromatography (HPLC) in a water/acetonitrile gradient containing 0.1 vol% NH_4OH to aid solubility and separation. The purified PAs were again rotary evaporated to remove the acetonitrile, then dialyzed against water from a MilliQ water purification system operating at 18.2 MΩ. Finally the PAs were lyophilized and stored in cold, dry conditions until use.

2.3. Rheology

Oscillating rheology was used to quantify the time to gelation and final mechanical properties of the molecules. Each PA was dissolved in aqueous solution at 1% w/v and pH 7.5–8. This solution was mixed 1:1 with Hank's Buffered Saline Solution (HBSS Sigma), or 20 mM calcium chloride on the stage of a Paar Physica Modular Compact Rheometer (MCR) 300 (Graz, Austria). A 25-mm parallel plate configuration was used and the temperature was held constant at 21 °C. Storage (G') and loss (G'') moduli and complex viscosity (V^*) were measured at 3% strain and 4 Hz for the IKVAV PAs, at 0.1% strain and 4 Hz for the RGD PAs, and 0.5% strain and 100 Hz for the EEE PAs, in order to carry out measurements in the linear viscoelastic region for each set of molecules. For time tests, measurements began 50 s after mixing due to the requirements of instrument setup. Final storage and loss moduli were measured at 4000 s after the start of measurement.

2.4. Circular dichroism

Circular dichroism measurements were carried out to correlate changes in gelation kinetics with the development of secondary structure. Wavelength scans were taken of the IKVAV-bearing PAs in water and 1:1 water:HBSS at 0.1 nm intervals between 250 nm and 185 nm at a concentration of 0.5 mg/mL with a 0.1 cm path length quartz cuvette. For the RGD PAs, measurements were taken in water and in 50 mM $CaCl_2$. For the EEE PAs, measurements were taken in water and in 20 mM $CaCl_2$. All samples were allowed to equilibrate for 2–3 h prior to measurement. The percentages of secondary structures were estimated by averaging the results of matrix-based data decomposition with CONTIN, SELCON, and CDSSTR algorithms using two distinct sets of basis vectors. CDPro software [26] was used for these calculations. Measurements were taken on a Jasco J-715 circular dichroism instrument at room temperature using a 1 mm cuvette. Data was taken at a resolution of at least 0.5 nm and averaged over at least 10 acquisition cycles.

2.5. Substrate preparation for cell studies

12-mm round glass coverslips (Carolina Biological) were distributed into 24-well cell culture plates (VWR). For the PA substrates, dilute (0.1 mg/mL) PA solutions were mixed 1:1 with HBSS and pipetted onto the coverslips in 100 μL aliquots. The solutions were allowed to dry overnight in a laminar flow hood under ambient conditions, after which the coverslips were rinsed once by pipetting 500 μL of sterile distilled water into each well, and aspirating the water to remove excess salt. Poly-D-lysine (PDL) and PDL-laminin were used as control substrates. For the PDL substrates, 500 μL of 0.1 mg/mL aqueous PDL (<300 kDa, Sigma) solution was pipetted onto the coverslips. After 2 h, the excess PDL was aspirated and the coverslips were rinsed three times with sterile distilled water to remove soluble polymer. The PDL-laminin substrates were treated identically, then 1 μg/mL laminin in PBS was added after the final water rinse. These substrates were also allowed to dry overnight.

2.6. Cell culture

Neural tissue was harvested from the lateral and medial ganglionic emminences of E13 mouse embryos, dissociated, and plated in Petri dishes. Neural progenitors were separated from other neural cell types by growing them as neurospheres through two passages as previously described [25,26]. Briefly, the lateral ganglionic eminence was dissected, triturated and grown in suspension using non-adherent flasks (Petri flasks, VWR) containing 45 ml of neural stem cell medium (DMEM/F12 (Gibco), 10 ml B27 supplement (Gibco), 5 ml N2 medium (Gibco), 5 ml of 100× Penicillin/Streptomycin/Glutamine solution (Gibco), 0.5 ml 2 mg/ml heparin (Sigma) and 20 ng/ml EGF (BD Bioscience)) at 37 °C and 5% CO_2. After 3 days, the spherical cell clumps (neurospheres) were dissociated by centrifugation and incubation with trypsin/EDTA, quenching with trypsin inhibitor, and triturating through a pipette tip. Cells were plated in PA gels at a density of 5×10^5 cells/ml.

After plating, the cells were incubated at 37 °C, 95% humidity, and 5% CO_2 for three days, after which cell viability was determined using calcein and ethidium homodimer-1 stains (Molecular Probes Live/Dead kit). The differentiation state of the cells was also assayed at three days by immunolabeling fixed coverslips with antibodies against the neuronal marker β-tubulin and glial marker glial fibrillary acid protein (GFAP) using a modification of the procedure used by Silva et al. [5]. Fluorescent micrographs were acquired at 200× magnification and the NIH-developed software program ImageJ (http://rsb.info.nih.gov/ij/) was used in the counting of the immunopositive cells.

2.7. Turbidity

Turbidity (absorption) was measured over time using a Cary 500 UV–vis spectrophotometer. Solutions of each PA at 0.5% by weight were mixed with an equal volume of HBSS in a 10-mm path length quartz cuvette and monitored for 60 min at 10 s intervals. Measurements were obtained at 600 nm.