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Modulated growth, stability and interactions of liquid-like coacervate assemblies of elastin

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ABSTRACT

Elastin self-assembles from monomers into polymer networks that display elasticity and resilience. The first major step in assembly is a liquid–liquid phase separation known as coacervation. This process represents a continuum of stages from initial phase separation to early growth of droplets by coalescence and later "maturation" leading to fiber formation. Assembly of tropoelastin-rich globules is on pathway for fiber formation in vivo. However, little is known about these intermediates beyond their size distribution. Here we investigate the contribution of sequence and structural motifs from full-length tropoelastin and a set of elastin-like polypeptides to the maturation of coacervate assemblies, observing their growth, stability and interaction behavior, and polypeptide alignment within matured globules. We conclude that maturation is driven by surface properties, leading to stabilization of the interface between the hydrophobic interior and aqueous solvent, potentially through structural motifs, and discuss implications for droplet interactions in fiber formation.

1. Introduction

Elastin is an extracellular matrix (ECM) protein that imparts the elastic properties of stretch and reversible recoil to vertebrate tissues including major blood vessels, lung and skin. Elastin self-assembles from its monomeric precursor, tropoelastin, into fibrous polymer networks that have the capacity for large elastic extensions under low forces. Elastin-rich tissues such as the human aorta can sustain up to billions of cycles of stretch and recoil over a lifetime, displaying remarkable durability given the lack of appreciable elastin turnover within mature tissues (Shapiro et al., 1991; Davis, 1993; Keeley et al., 2002). The functional properties of elastin arise from the correct organization of monomers during polymer self-assembly that features a key phase separation step and are highly dependent on sequence and structural motifs. Despite many advances, the molecular events contributing to the assembly of fibers remain poorly characterized.

Tropoelastins across phylogeny share little overall sequence homology but retain specific sequence features characteristic of all elastins (Keeley, 2013). Tropoelastin is a highly (>75%) non-polar protein principally characterized by hydrophobic sequence blocks, called domains, of low sequence complexity that alternate with lysine-containing cross-linking domains. Human tropoelastin (~60 kDa) is comprised of 34 such domains (Fig. 1). Hydrophobic domains are rich in glycine, valine and proline residues that are commonly arranged into quasi-repetitive motifs such as VPG and PGVG (He et al., 2007). Despite their hydrophobic character, these sequences remain substantially disordered and flexible in solution, where they support the transient population of local β-turn and polyproline II helix structural motifs (Tamburro et al., 2003; Muiznieks et al., 2010). This is largely due to their high (~50%) combined composition of glycine and proline residues (Rauscher et al., 2006), as well as an average proline spacing of four to eight residues (Muiznieks and Keeley, 2010). These residues restrict the formation of extended secondary structures such as α-helix and β-sheet due to the high entropic cost for structural confinement of glycine, and the fixed phi dihedral angle and lack of backbone amide hydrogen-bond donor of proline. Conformational disorder is essential for elastin function, as returning to a state of higher entropy after extension is one of the driving forces for elastic recoil (Aaron and Gosline, 1981; Rauscher et al., 2006; Rauscher and Pomes, 2012).

Cross-linking domains contain lysine residues that are almost exclusively found in pairs spaced three or four residues apart. The formation of covalent cross-links by these lysine residues provides fibrous elastic networks with high resilience and structural integrity. Lysine residues in cross-linking domains are arranged either on a poly-alanine background

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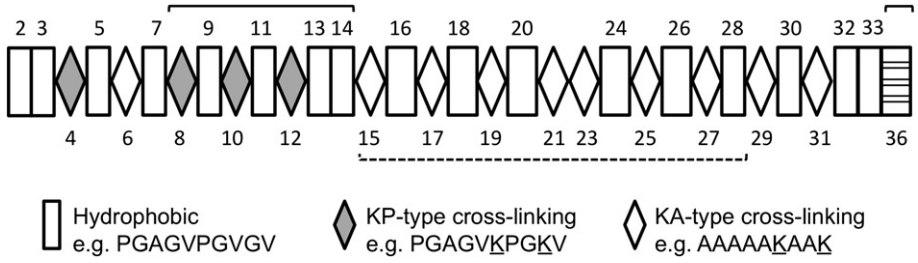

Fig. 1. Schematic domain arrangement of human tropoelastin. Hydrophobic domains (*open rectangles*) alternate with cross-linking domains of KP-type (*shaded diamonds*) or KA-type (*open diamonds*). Domains are labeled above and below. Representative sequence for each of these domain types is given. Motifs conserved across phylogeny are indicated by a solid bar. The C-terminal domain 36 (*horizontal stripes*) contains an RKRK motif. An expanded region that likely arose from the duplication of a KA-type cross-linking and hydrophobic exon pair is indicated by a dotted bar. Domain 22 is not observed from the most common isoform of mature tropoelastin. Genomic sequences corresponding to exons 34 and 35 have been lost in humans through evolution.

such as AAAAAKAAK (denoted KA-type), or within sequences rich in hydrophobic residues in the form of PGAGVKPGKV (denoted KP-type). The majority of cross-linking domains in mammalian and avian species are of the KA-type. These poly-alanine stretches display propensity for α-helix, although helical motifs remain unstable and/or not fully formed in solution (Muiznieks et al., 2003; Miao et al., 2005; Reichheld et al., 2014). In contrast, KP-type cross-linking domains remain largely disordered (Tamburro et al., 2003), consistent with their high (~50%) proline and glycine residue content.

Elastic fiber assembly is a hierarchical, multi-stage process. The first major step is a thermosensitive liquid–liquid phase separation, known as coacervation, that is characterized by the formation of a dense, protein-rich phase dispersed in a protein-poor solution phase (Yeo et al., 2011). This process is akin to the lower critical solution temperature (LCST) phase transition described for a range of polymers, which is driven by an unfavorable entropy of solvent–solute mixing (Van Vlierberghe et al., 2011). In the case of elastin, phase separation is attributed to the hydrophobic effect and thus associated with an increase in solvent entropy upon the burial of hydrophobic domains (Vrhovski et al., 1997). Coacervation behavior is observed in vitro from both full-length tropoelastin and smaller elastin-like polypeptides (ELPs) as a clear solution that becomes turbid upon reaching a critical transition temperature (Vrhovski et al., 1997; Bellingham et al., 2001). Many factors influence the onset of coacervation including polypeptide concentration and sequence features such as length, composition and domain arrangement, as well as solution temperature, pH and ionic strength (Vrhovski et al., 1997; Toonkool et al., 2001a; Miao et al., 2003). In vivo, this process is likely to be initiated by a rise in concentration concomitant with monomer secretion into a volume-restricted compartment (Kozel et al., 2006). Although coacervation is initially reversible, it is becoming apparent that a complex, spontaneous and irreversible maturation process follows the initial phase separation event, potentially guiding the growth, stabilization and interaction of the coacervate towards the formation of fibers (Cirulis et al., 2009; Naumann et al., 2009). However, the molecular details of this process are not well understood. In particular, the contributions of individual sequence elements to the stages of coacervate maturation remain unclear.

The phenomenon of solution turbidity arises from the scattering of light by particles. We and others have shown that phase-separated coacervate particles correspond to colloid-like droplets in solution that typically grow by coalescence (Kaibara et al., 2000; Clarke et al., 2005, 2006; Cirulis et al., 2008). The formation of spheroidal droplets is consistent with the minimization of hydrophobic surface area exposed to solvent (Pappu et al., 2008). Similar tropoelastin-rich globules of roughly 1–6 μm diameter are a feature of elastogenesis in vivo (Kozel et al., 2004, 2006). Assemblies are anchored to the outer cell surface before being incorporated into fibrillar architectures, strongly suggesting a mechanistic role for these coacervate-like globules in the transport and deposition of tropoelastin to microfibrils during elastic fiber assembly. Coordinated cell motions and ECM deformations exert a dominant influence on the progressive aggregation of tropoelastin-rich globules in vivo (Czirok et al., 2006; Kozel et al., 2006). Additionally, ECM macromolecules affect coacervate droplet growth (Cirulis et al., 2008) and lower the critical monomer concentration required for droplet formation in vitro (Clarke et al., 2005; Tu and Weiss, 2008, 2010). However, modulators of the biophysical properties and association behavior of these colloidal assemblies are not well described.

Here we monitor the growth, stability and interaction behavior of colloidal droplets formed from a range of ELPs and tropoelastin and detail specific sequence and structural motifs that contribute to the differential stability and morphology of the macromolecular assemblies. Together, these data suggest that stabilization of coacervate assemblies proceeds via the formation of secondary structure at the droplet surface. These results highlight the requirement for control over coacervate droplet growth and stability for proper fiber assembly and offer a model for coacervate droplet interactions in fiber formation. Moreover, an understanding of how sequence and structural features affect droplet morphology and stability will be important for the rational design of stimulus-responsive ELP nanoparticles for biomedical drug delivery applications.

2. Results

2.1. Phase separation of elastin: formation and general behavior of the coacervate

Phase separation is an intrinsic property of elastin that is largely dependent on hydrophobic domain content including number, length and juxtaposition with cross-linking domains, and strongly modulated by solvent environment including ionic strength, pH and temperature (Vrhovski et al., 1997; Bellingham et al., 2001; Miao et al., 2003). In this study we monitored the progression of elastin phase separation from early onset to the formation of matured architectures by light microscopy using a range of model elastin-like polypeptides and full-length tropoelastin. For each sequence, the onset of solution turbidity (coacervation) was observed as the rapid formation of droplets once the solution temperature was raised above the coacervation temperature. In the first few minutes after the initial phase separation event, particles grew rapidly by coalescence independent of the polypeptide sequence, indicating predominant surface instability of the droplets dispersed within the emulsion. Coalescence was a diffusion-limited process, resulting in polydisperse (non-uniformly sized) droplets in solution over time. As the droplets grew large enough to respond to gravitational forces they began to settle in solution towards the base of the sample chamber indicating instability of the suspension. However, in no case did the protein-rich droplets fully separate into a single continuous phase. Moreover, no reduction in droplet sizes was observed, implying no redissolution of droplets or equilibrium between elastin in the droplet and solution phase.

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