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Elastin as a self-organizing biomaterial: use of recombinantly expressed human elastin polypeptides as a model for investigations of structure and self-assembly of elastin

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Elastin is the major extracellular matrix protein of large arteries such as the aorta, imparting characteristics of extensibility and elastic recoil. Once laid down in tissues, polymeric elastin is not subject to turnover, but is able to sustain its mechanical resilience through thousands of millions of cycles of extension and recoil. Elastin consists of *ca*. 36 domains with alternating hydrophobic and cross-linking characteristics. It has been suggested that these hydrophobic domains, predominantly containing glycine, proline, leucine and valine, often occurring in tandemly repeated sequences, are responsible for the ability of elastin to align monomeric chains for covalent cross-linking. We have shown that small, recombinantly expressed polypeptides based on sequences of human elastin contain sufficient information to self-organize into fibrillar structures and promote the formation of lysine-derived cross-links. These cross-linked polypeptides can also be fabricated into membrane structures that have solubility and mechanical properties reminiscent of native insoluble elastin. Understanding the basis of the self-organizational ability of elastin-based polypeptides may provide important clues for the general design of self-assembling biomaterials.

Keywords: elastin; self-assembly; recombinant elastin polypeptide; coacervation; cross-linking

1. INTRODUCTION

Elastin is the major extracellular matrix protein in tissues such as the large arterial blood vessels, lung parenchyma, elastic ligaments and skin, where it is accepted to be principally responsible for the physical properties of extensibility and elastic recoil that are particularly important for the function of these tissues. Elastin is also the major matrix protein in some cartilaginous tissues such as ear cartilage, where the functional role of this protein is less evident.

In common with most structural proteins elastin is synthesized as a monomer, tropoelastin, which is subsequently assembled into a stable, polymeric structure in the extracellular matrix (Mecham & Davis 1994; Vrhovski & Weiss 1998). This polymer is stabilized by covalent cross-links formed through interactions between side chains of lysine residues after oxidative deamination by lysyl oxidase. Polymeric elastin has the unusual property of being essentially insoluble in all reagents except those which break polypeptide bonds, and this persistent insolubility of elastin has been both a benefit in the isolation and purification of the protein and an impediment to its structural characterization. In addition, with the notable exception of the uterus (Starcher & Percival 1985), elastin, once laid down in its insoluble polymeric form in the extracellular matrix of tissues, does not turn over at any appreciable rate under normal circumstances (Davis 1993). In effect, this means that the elastin present in the aorta of an older human is the elastin that was laid down during aortic development. The fact that this aortic elastin has literally gone through thousands of millions of cycles of extension and recoil without mechanical failure is a testament to the remarkable properties of elastin as a biomaterial.

For more than three decades there has been a continuing lively debate over the basis for the elastomeric properties of this protein. Attempts to account for the elastomeric properties of elastin through structural models have a long history, from the earlier globular models of Partridge (1966), to the 'oiled coil' model of Gray *et al.* (1973), to the 'librational motion' model of Urry (1988). As alluded to earlier however, one of the major impediments to detailed structural analysis of elastin is the insolubility of the protein, limiting at least some of the most powerful approaches such as X-ray crystallography and solution NMR. The more recent availability of monomeric elastin has not been of particular assistance in this respect because of the strong tendency of this monomer for self-aggregation, of which more will be said later. Many structural studies have used circular dichroism and other physical techniques, either on hydrolytic fragments of insoluble elastin or on synthetic model peptides based

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on elastin sequences (Urry *et al.* 1969; Jamieson *et al.* 1972; Urry 1988; Tamburro *et al.* 1992; Castiglione Morelli *et al.* 1993; Reiersen *et al.* 1998; Debelle *et al.* 1998). Alternatively, structural conclusions have been drawn from various techniques used to image the polymeric elastin matrix (Ross & Bornstein 1969; Pasquali Ronchetti *et al.* 1979; Bressan *et al.* 1986; Mecham & Heuser 1991). More recently, computational methods have also been used to predict the structure of specific domains of elastin (Debelle *et al.* 1998).

Leaving the thermodynamics and biomechanics arguments to others, there can be agreement on some fundamentals. First, as data are becoming available on more elastomeric proteins, it is clear there is no obvious single protein sequence that confers properties of elastic recoil on these proteins, although similarities in protein design may be very informative. Second, the way in which the monomers are organized into polymeric structures must be crucial for their elastomeric properties. It is this question of the organization of elastin monomers into polymeric elastin that we will deal with here.

Unlike collagen, tropoelastin does not have 'registration' or propeptides at N- or C-terminals of the polypeptide, which aid in the alignment of monomers for polymer formation. However, several other components have been suggested to contribute to the organization of the elastin polymer. These include the proteins that form the microfibrillar scaffolding in many elastic tissues, based on early observations, that during development of elastic tissues newly synthesized elastin appears to coalesce on a preformed bed of microfibrills in the extracellular matrix (Ross & Bornstein 1969; Ross *et al.* 1977). Proteins comprising this microfibrillar scaffolding include the fibrillins, which themselves have some intrinsic properties of extensibility and elastic recoil (McConnell *et al.* 1996; Baldock *et al.* 2001). More recently, an elastin-binding protein has been suggested to be important both for the intracellular chaperoning of monomeric elastin and for the presentation and transfer of tropoelastin on the cell surface to the growing elastin fibre (Hinek *et al.* 1988; Hinek & Rabinovitch 1994). Lysyl oxidase may also have an organizing role in elastin polymer formation above and beyond simply oxidatively deaminating lysine side chains in preparation for cross-linking.

Several years ago Bressan *et al.* (1986) suggested that the inherent ability of elastin to self-aggregate might be an important factor contributing to the polymeric organization of elastin. That is, the formation of these aggregates played an organizational role in the alignment of tropoelastin monomers for polymeric assembly and cross-link formation. Self-aggregation of elastin was first described as a coacervation phenomenon, in which hydrophobic proteins come out of solution as a second phase on an increase in solution temperature. Coacervation of elastin was originally described for heterogeneous mixtures of hydrolytically solubilized insoluble elastin (α elastin) (Partridge *et al.* 1955; Urry *et al.* 1969; Jamieson *et al.* 1972), but was subsequently shown to also take place with synthetic peptides based on hydrophobic domains in elastin (Urry 1988; Tamburro *et al.* 1992; Castiglione Morelli *et al.* 1993; Reiersen *et al.* 1998), as well as in the full-length tropoelastin molecule (Vrhovski *et al.* 1997). The kinetics of coacervation of elastin are similar to those of a nucleation process.

In recent years there has been renewed interest in the mechanisms of assembly of elastin, spurred particularly by the technology for expression of recombinant proteins. We have used a 'minimalist' approach to understanding the assembly of elastin and the relationship between the organization of elastin polymers and the structural and mechanical properties of the elastin polymer. That is, we have attempted to establish the minimal sequence requirements for self-organization and assembly of elastin monomers into polymeric matrices with elastin-like properties. Although this work is still in its early stages, the data shown here clearly indicate that even rather small polypeptides based on elastin domains not only have the ability for directed and organized self-assembly, but also result in matrices with physical properties similar to elastin.

2. PRODUCTION AND CHARACTERIZATION OF RECOMBINANT ELASTIN POLYPEPTIDES (EPs)

(a) *Recombinant expression and purification*

Details of the design, recombinant expression and isolation of the EPs are given elsewhere (Bellingham *et al.* 2001). Briefly, polypeptides based on sequences in human elastin were produced by a GST fusion protein approach using the pGEX-2T vector (Amersham Pharmacia Biotech, Inc., Baie d'Urfe, Quebec, Canada). Human elastin cDNA sequences for insertion into the pGEX-2T vector were obtained by PCR using clones as templates (a gift of Dr Charles Boyd, Pacific Biomedical Research Center, University of Hawaii). Constructs included a methionine residue placed just upstream of the N-terminal of the elastin peptide sequence. GST fusion proteins were concentrated by an affinity method using glutathione–agarose, and cyanogen bromide was used to release the elastin polypeptide from the fusion protein, followed by purification by chromatographic methods. Because of the absence of internal methionine residues, the EPs remain intact after cyanogen bromide treatment. Purity of the recombinant polypeptides was confirmed by amino acid analysis and protein mass spectrometry.

Details of the EPs are shown in figure 1. All polypeptides included at least one cross-linking domain of elastin, consisting of exons 21 and 23. These are classical cross-linking sequences of elastin containing lysine residues on a polyalanine background, with pairs of lysines separated by either two or three alanine residues. Except for EP 21–23, which contains only the cross-linking domains, EPs were designated according to their hydrophobic exons. Thus, EP 20–24 contains exons 20–21–23–24 and EP 20–24–24 contains exons 20–21–23–24–21–23–24. Exon 20 and 24 are hydrophobic exons of elastin, with exon 24 containing the most striking polypeptide repeat of human elastin, consisting of seven tandem repeats of a PGVGVA motif. The sequences of these exons are illustrated in figure 1 *a*,*b*.

(b) *Coacervation and oxidative deamination of EPs*

Coacervation characteristics of the EPs were determined by a spectrophotometric method described in detail elsewhere (Bellingham *et al.* 2001). Coacervation was

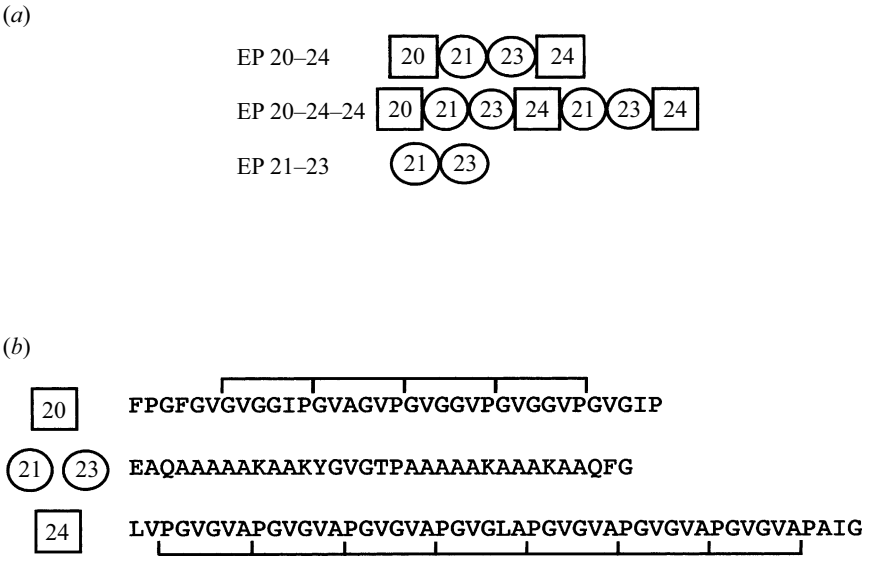

Figure 1. Recombinant polypeptides based on sequences in human elastin. Polypeptides were produced as GST fusion proteins, using a methionine residue placed just upstream of the first amino acid of the elastin sequence as a site for cleavage with cyanogen bromide. The released EPs were purified by ion exchange and gel filtration chromatography. Polypeptides contained exons 20, 21, 23 and 24 in combinations illustrated in (*a*). Sequences of these exons are illustrated in (*b*). Exons 20 and 24 are hydrophobic domains with tandem repeats. Exons 21 and 23 together form a classic cross-linking domain containing two pairs of lysine residues separated by either two or three alanine residues.

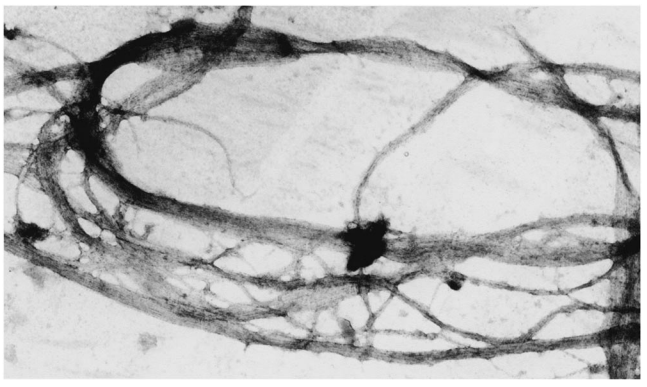

Figure 2. Spontaneous formation of fibrillar matrices by EP 20–24–24 after coacervation. The polypeptide was dissolved in coacervation buffer (25 μm), and the solution temperature slowly increased until coacervation took place. The solution temperature was maintained at 39 °C for 16 h, after which time the coacervate was layered onto prewarmed copper grids and stained with 2% aqueous uranyl acetate for TEM.

induced by increasing the solution temperature at a rate of 1 °C min^{-1}. The onset of coacervation was detected by a sudden increase in turbidity of the solution, assessed by monitoring absorbance at 440 nm.

Elastin peptides were oxidatively deaminated either using a catechol/peroxidase reagent (Stahmann *et al.* 1977) or by treatment with a solution of PQQ in the presence of cupric sulphate (Shah *et al.* 1992). Amino acid analyses before and after oxidation confirmed that only lysine residues of the polypeptides were altered by this treatment. The identification of desmosine and isodesmosine cross-links in the polypeptides aggregates utilized a radioimmunoassay method specific for these cross-links (King *et al.* 1980; Starcher *et al.* 1995).

(c) *Characterization and mechanical testing of matrices fabricated from EPs*

Matrices produced from EPs were subjected to overnight extraction by cyanogen bromide (50 mg ml^{-1} of CNBr in 70% formic acid) at room temperature, or treatment with 0.1 M of NaOH at 100 °C for 45 min. The mechanical properties of matrices were determined on an Instron tensile testing apparatus in the laboratory of Dr John Gosline, University of British Columbia.

3. SELF-ASSEMBLY AND POLYMERIZATION OF RECOMBINANT POLYPEPTIDES

As expected, with the exception of EP 21–23, which contained no hydrophobic domains, these recombinant EPs had the ability to coacervate, confirming the requirement for hydrophobic domains. As had previously been reported for tropoelastin (Vrhovski *et al.* 1997), the temperature of coacervation was inversely related, both to the concentration of the polypeptides and to the ionic strength of the coacervation solution. Equimolar concentrations of EP 20–24–24 coacervated at a significantly lower temperature (*ca.* 30 °C) as compared with EP 20–24 (*ca.* 41 °C). These results confirmed that polypeptides containing both cross-linking and hydrophobic domains, but representing as little as 15% of the entire tropoelastin monomer, possessed the ability to self-aggregate. Preliminary data (not shown) have suggested that the propensity of these polypeptides to self-assemble, as measured by coacervation temperature, is related not only to their hydropathy and molecular weight but also to the specific hydrophobic sequences they contain. We are currently investigating the details of the relationship between specific hydrophobic sequences, tandem sequence repeats and the capacity of these polypeptides for self-aggregation.

The ability of these polypeptides to aggregate is, in

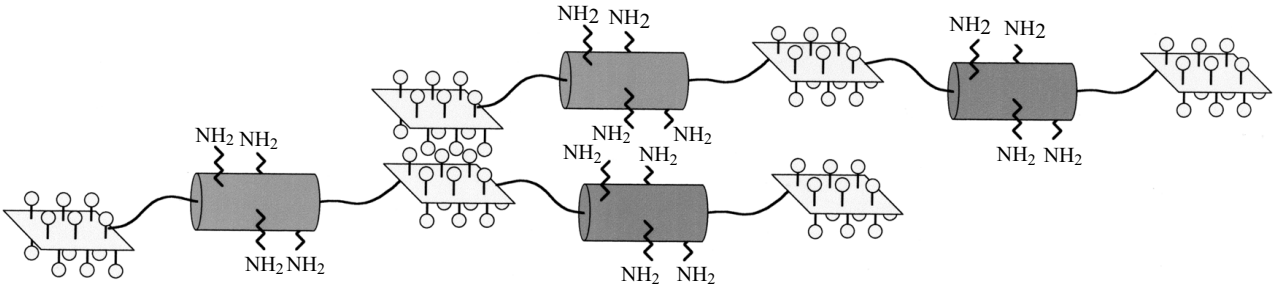

Figure 3. Representation of the postulated alignment of EPs during coacervation, juxtaposing side chains of lysine residues for subsequent formation of covalent cross-links after oxidative deamination of lysine side chains. Hydrophobic domains are represented by square planar structures with large hydrophobic side chains protruding above and below the plane ('lego motifs'), allowing interactions by interdigitation of hydrophobic side chains. Cross-linking domains are represented by cylinders, with pairs of lysine residues protruding on the same side of the α-helical structure. Formation of desmosine or isodesmosine cross-links requires condensation of four lysine side chains, three of which have been oxidatively deaminated to lysine aldehydes.

itself, a relatively uninteresting property unless self-association, as postulated earlier by Bressan *et al.* (1986), results in an alignment of these monomers, which contributes to the structural organization of the polymer. Evidence for the formation of orientated, fibrillar structures on coacervation of tropoelastin (Cox *et al.* 1973; Urry *et al.* 1974; Bressan *et al.* 1986) had supported the view that self-aggregation involved a specific spatial organization of the elastin monomers. Similarly, electron microscopy of coacervates of EP 20–24 and EP 20–24–24 has demonstrated that both of these polypeptides were capable of the formation of fibrillar structures on self-aggregation similar to those reported for tropoelastin (figure 2).

The formation of fibrillar structures on self-aggregation, either of these polypeptides representative of tropoelastin or of the whole tropoelastin molecule, only implies a rather limited level of structural organization of these monomers during self-assembly. A much more significant test of the self-organizational ability of these monomers is whether, as a consequence of the association of the hydrophobic domains, lysines in the cross-linking domains of these polypeptides are aligned such that cross-linking can take place (figure 3). Earlier data (Bedell-Hogan *et al.* 1993) had indicated that oxidative deamination of recombinant tropoelastin using lysyl oxidase resulted in the formation of covalent, lysine-derived cross-links. Here we used either a catechol/peroxidase reagent, or a PQQ/copper redox system to oxidize lysine residues in the polypeptides. After either method, amino acid analysis confirmed oxidative deamination of *ca.* 80% of the lysine residues, with no other amino acid affected. In the absence of coacervation, although lysine residues in the polypeptides were oxidatively deaminated, no significant cross-linking of the polypeptides took place. In the absence of oxidation of lysines, aggregates of polypeptides were formed by coacervation, but these aggregates could be completely solubilized in solutions of 2% SDS/4 M urea. In contrast, coacervation of polypeptides in which lysines were oxidatively deaminated formed aggregates that were insoluble in 2% SDS/4 M urea. Moreover, lysine-derived cross-links, including both desmosine and isodesmosine, the major naturally occurring covalent cross-links in insoluble elastin, were identified in these aggregates. These results demonstrated that the process of self-aggregation

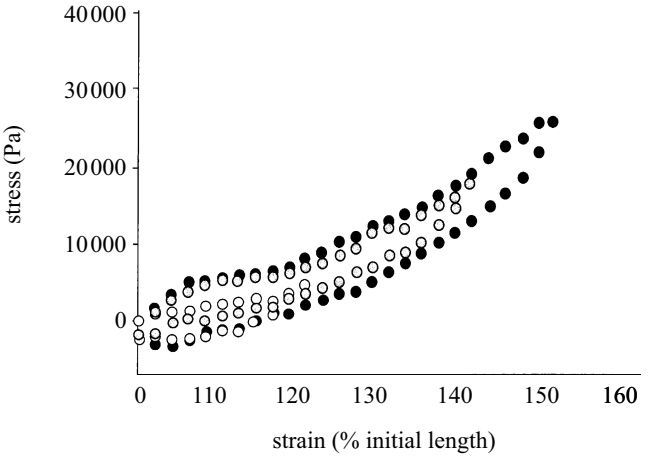

Figure 4. Stress–strain curves for membrane structures fabricated from EP 20–24–24 after coacervation followed by oxidative deamination of lysine side chains with PQQ. These stress–strain curves represent three cycles of loading and unloading, with the length of extension progressively increased. The breaking strain is at *ca.* 100% of the initial length. Young's modulus of these membranes is *ca.* 0.2 MPa. Open circles, first cycle (20% extension); grey circles: second cycle (40% extension); black circles, third cycle (50% extension).

of these polypeptides involved the organizational alignment of the monomers such that zero-length cross-linking involving oxidized lysines could take place.

Fabrication of these covalently cross-linked aggregates of recombinant EPs into membrane-like matrices has allowed further assessment of their solubility characteristics and physical properties. Like insoluble elastin, these matrices are resistant both to treatment with cyanogen bromide and to extraction with hot 0.1 M of NaOH. Preliminary mechanical testing of these matrices indicates physical properties which are remarkably similar to those of native insoluble elastin, including comparable elastic moduli, extensibility and elastic recoil characteristics (figure 4), and similar strains to failure.

These data strongly support the view that monomers of elastin possess the inherent ability to organize themselves into polymeric structures, aligning lysine residues for

subsequent cross-link formation. Furthermore, this ability for self-alignment can be mimicked in EPs containing small numbers of hydrophobic and cross-linking domains. Thus, while the presence of the microfibrillar scaffolding, the chaperoning role of the elastin-binding protein and the action of lysyl oxidase are all, without doubt, important for establishing the appropriate architecture of insoluble elastin in tissues, it appears that the fundamental requirements for organization of monomeric elastin into a matrix, with properties of extensibility and elastic recoil, can be provided by the intrinsic self-organizational ability conferred by the presence of a few domains of the elastin monomer and the presence of an oxidizing agent. Such recombinant polypeptides therefore provide a useful tool for the investigation of the role of the polypeptide sequence and domain organization in determining the structural organization and material properties of insoluble elastin.

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