Published in final edited form as:

Chem Commun (Camb). 2010 January 14; 46(2): 216–218. doi:10.1039/b915775a.

Spontaneous and reversible self-assembly of a polypeptide fragment of insulin-like growth factor binding protein-2 into fluorescent nanotubular structures†

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Abstract

In this communication, we report the spontaneous and reversible *in vitro* self-assembly of a polypeptide fragment derived from the C-terminal domain of Insulin-like Growth Factor Binding Protein (IGFBP-2) into soluble nanotubular structures several micrometres long *via* a mechanism involving inter-molecular disulfide bonds and exhibiting enhanced fluorescence.

In recent years, self-assembly of peptides to form nano-structured materials has emerged as an important area of research.^{1,2} In general, non-covalent interactions within or between the peptide building blocks have been the prime focus for understanding self-assembly.^{1,2} We report here a novel self-assembly process involving covalent, inter-molecular disulfide bonds observed in a polypeptide fragment of Insulin-like Growth Factor Binding Protein (IGFBP-2). IGFBP-2 belongs to the IGF system which plays an important role in the growth and function of the human body.³ This system is comprised of peptide hormones, IGF-1 and IGF-2, which are regulated by six soluble IGFBPs (numbered 1–6). IGFBPs range from 22 to 31 kDa in size and share overall sequence and structural homology with each other.^{4,5} In our laboratories, we have undertaken structural and functional studies of human IGFBP-2 as a model system.⁶ In studying different truncation mutants of hIGFBP-2 and their interaction with IGF, we focused on a polypeptide fragment derived from the 41 amino acid residue C-terminus of the protein (residues 249–289; hIGFBP-2_{249–289}), downstream of its conserved CWCV motif and containing the RGD motif.⁶ This poly-peptide stretch was found to be largely unstructured in the three-dimensional (3D) structure of the C-terminal domain of hIGFBP-2 (Fig. 1(a)).⁷ While the native form of hIGFBP-2_{249–289} has two cysteines (C-249

†Electronic supplementary information (ESI) available: Sample preparation, TEM, SDS-PAGE and NMR.

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and C-270), the polypeptide fragment that we cloned and expressed had an additional cysteine at position 281 (Fig. 1(a)).⁸

Under reducing conditions such as in the presence of 1 mM β -mercaptoethanol at neutral pH the protein remained as a monomer. However, upon removal of β -mercaptoethanol by dialysis and/or ultrafiltration, it spontaneously self-assembled into soluble nanotubes, several micrometres long that were detectable by fluorescence microscopy. We report here the discovery and characterization of these nanotubes carried out using transmission electron microscopy (TEM), nuclear magnetic resonance (NMR) spectroscopy and circular dichroism (CD). Based on the results obtained, we propose a model for their formation. The observation that formation/dissociation of these nanotubes can be controlled at will, their high stability due to covalent interaction between the individual components and fluorescent properties offers new avenues for designing novel, IGFBP-based self-assembling nanotubes.

Fig. 1(b) and (c) show the TEM images of hIGFBP-2_{249–289} samples prepared in the absence of β -mercaptoethanol (the details of protein production, sample preparation and experimental parameters are described in the ESI[†]). Nanotube formation was also monitored by SDS-PAGE and gel-filtration, wherein their mobilities were equivalent to high molecular weight proteins.

Under non-reducing conditions, the nanotubes form by self-assembly due to the presence of active cysteine groups (free sulfhydryls) which link covalently *via* disulfide bonds (a model for formation of the tubular structures is described below). Upon addition of β -mercaptoethanol, they disassemble into monomer units (verified by NMR) due to reduction of the disulfide bonds and thus are not visible in the TEM images (Fig. S1, ESI[†]). Similarly, the nanotubes lose their fluorescence properties as monomers, presumably due to a loss in signal intensity from the single tyrosine present. The formation/dissociation of the nanotubes was repeated several times and was always found to be a reversible process and independent of the concentration of the monomer. The nanotubes were formed within 2–3 h of removal of β -mercapto-ethanol at 25 °C for a range of concentrations. To test the stability of the protein at high temperature, the sample containing nanotubes was heated to 95 °C. The presence of high molecular weight bands in SDS gels (see Fig. S2, ESI[†]) indicated that the nanotubes remained intact thus displaying high thermal stability.

We also carried out a detailed NMR spectroscopic investigation of the monomers and nanotubes in order to define site-specific structural modifications occurring upon self-assembly. Fig. 2 shows the two-dimensional (2D) [¹⁵N, ¹H^N] HSQC⁹ spectra of the monomer and the nanotube, which were assigned sequence-specifically using standard NMR techniques⁹ (details of sample preparation and NMR experiments are provided in the ESI[†]).

An overall rotational correlation time of ~3 ns (measured using ¹⁵N T_1 and T_2 relaxation rates)⁹ was obtained for the sample prepared with β -mercaptoethanol, indicating that the polypeptide existed as a monomer under reducing conditions. On the other hand, the absence

[†]Electronic supplementary information (ESI) available: Sample preparation, TEM, SDS-PAGE and NMR.

of monomers in the sample containing the nanotubes was confirmed by mass spectrometry and SDS-PAGE.

In the monomeric state, the polypeptide fragment is largely unstructured (Fig. 1(a)) which is also evident from: (i) the low spectral dispersion seen in the 2D HSQC spectrum (Fig. 2(a)) and (ii) the secondary shifts calculated for the backbone ^1H and ^{13}C shifts⁹ and (iii) CD spectrum (Fig. S3, ESI[†]). In the nanotubular form, the rotational correlation time of the molecule increases due to an increase in molecular mass and thus peaks belonging only to residues that are structurally flexible are observed in the NMR spectrum. As shown in Fig. 2(b), approximately 50% of the peaks were broadened beyond detection. The $^1\text{H}^{\text{N}}$ and ^{15}N chemical shifts of the peaks belonging to structurally flexible residues in the monomers and nanotubes did not change significantly for most of the amino acid residues. Some propensity to form a β -sheet upon self-assembly was evident in the NMR spectra acquired using the method of CCSI-PRO¹⁰ (Fig. S4, ESI[†]). However, the precise location of secondary structural elements in the nanotubes could not be determined due to incomplete NMR assignments arising from line broadening. The chemical shifts of the three cysteine residues were investigated by 3D CBCA(CO)NH experiment to monitor the presence/absence of disulfide bonds. In the monomeric state, all cysteines exist in the reduced state as indicated by their $^{13}\text{C}^{\beta}$ chemical shift of ~ 28 ppm, whereas the presence of the disulfide bonds in nanotubes was reflected in their $^{13}\text{C}^{\beta}$ shifts of ~ 41 ppm.¹¹ This was further confirmed in the CD spectrum (data not shown) wherein a strong absorption at 240–250 nm, indicated the presence of disulfide bonds.¹²

Since the polypeptide contains a single tyrosine residue, fluorescence emission spectra were acquired using an excitation wavelength of 280 nm (Fig. 3). Notably, the nanotubes show higher intensity of tyrosine fluorescence emission as compared to the monomer (Fig. 3) indicating an increase in intrinsic fluorescence upon self-assembly. This allowed the imaging of the nanotubes by fluorescence microscopy (Fig. 1(d)). Such intrinsic fluorescence enables monitoring of the formation/dissociation of nanotubes in solution.

Self-assembly of polypeptides into nanotubes begins with a nucleation site such as the formation of a basic unit or a building block followed by non-covalent/covalent interaction between the component units resulting in tubular structures.^{1,2} In order to estimate the building blocks in hIGFBP-2_{249–289} and the nature of the interaction between them leading to nanotubes, we carried the following additional experiments: (i) heating nanotubes to high temperature and (ii) mutating the extra cysteine (C-281) to arginine as seen in wild-type hIGFBP-2. First, the formation of monomers under reducing conditions directly indicates the presence of disulfide bonds in the formation of nanotubes. However, there are two possibilities for the disulfide bonds to exist: (i) within the oligomeric basic unit and/or (ii) between the units. The former was verified by addition of β -mercaptoethanol resulting in the monomer, while the latter was verified by the fact that heating the nanotube sample to 95 °C retains the tubular structure (see Fig. S2, ESI[†]). The polypeptide fragment wherein the third cysteine (C281) was mutated to arginine as in wild-type hIGFBP-2⁷ did not form nanotubes even under non-reducing conditions (see Fig. S5, ESI[†]). This implies that the extra cysteine is instrumental for self-assembly. However, if only one out of the three cysteines is involved, at most one would generate a dimer. Formation of such ordered tubular structures

is expected to take place only when all the cysteines participate. Based on these observations, we propose a model for self-assembly (Fig. 4). Under non-reducing conditions, an oligomeric unit/building block forms first by intermolecular disulfide bonds between the polypeptide fragments. Such oligomers then connect to one another to form long tubular structures by disulfide bonding. Furthermore, the fact that we see a single set of peaks in the NMR spectrum and that a uniform diameter for all the tubular structures is observed in the TEM implies that a unique disulfide bonding is preferred over all other possible S–S bonding.

In recent years, a number of novel polypeptide and DNA based nanotubes have been reported.¹³ In one study, cysteines were engineered at appropriate positions within a protein, and the individual units were shown to link together by disulfide bonds into nanotubular structures.¹⁴ However, in all these studies, the oligomer forming the basic unit of self-assembly was comprised of protein subunits linked to each other by non-covalent interactions. Further, nanotubes in general are rendered fluorescent by attaching an external fluorescent chemical moiety. The present study reveals intrinsically fluorescent self-assembling nanotubes made up of disulfide bonds. This has the following advantages for nanotube design: (i) their formation/dissociation can be controlled by tuning the redox conditions; (ii) they do not require the support of any additional chemical agent for self-assembly; (iii) they have high stability due to the involvement of covalent interactions and (iv) the monomer is a small polypeptide chain which can be chemically synthesized or produced using simple recombinant methods.

In conclusion, we report for the first time that a polypeptide fragment from an IGFBP self-assembles spontaneously and reversibly into nanotubular structures. This has several potential applications. Engineering a different number of cysteines along a polypeptide chain offers a way to stitch the building blocks into stable and reversible nanotubular structures of different diameters. In addition, the presence of an RGD motif in this polypeptide fragment offers avenues for novel biomedical applications. The RGD motif is known to be recognized by integrins.¹⁵ The design of such self-assembling polypeptide fragments containing an RGD motif can be utilized to enhance the efficacy of cancer therapeutics such as those involving targeted drug delivery.¹⁶ Work in this direction is currently in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by research grants from Association of International Cancer Research, UK and NIH grants R01CA78887 and R01CA134845. Support from the NMR facility at NMR Research Centre (IISc) funded by Department of Science and Technology, India, Nanoscience Centre and Department of Organic Chemistry (IISc) is gratefully acknowledged. M. S. acknowledges a fellowship from CSIR, India.

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Fig. 1.

(a) 3D structure of the C-terminal domain of hIGFBP-2 (PDB code: 2h7t). The C-terminal fragment (residues 249–289) used in the current study is shown in yellow. The two cysteine residues in native hIGFBP-2 (pink) and the additional cysteine in place of an arginine in the fragment expressed in the current study (R281C) is indicated. (b) and (c) TEM images of hIGFBP-2_{249–289} prepared in the absence of β -mercaptoethanol. A magnified portion of the image is shown in the inset in (c) indicating an outer diameter of 35 nm for the tubular structures. (d) Fluorescence microscope image of hIGFBP-2_{249–289} nanotubes.

Fig. 2.

(a) and (b) 2D $[^{15}\text{N}, ^1\text{H}]$ HSQC spectra of IGF1BP-2249-289 under reducing (monomer) and oxidizing (nanotube) conditions, respectively. The sequence-specific resonance assignments are indicated on each peak by the single letter amino acid code followed by the residue number.

Fig. 3.
Fluorescence emission spectra of IGFBP-2_{249–289} monomer (black) and nanotube (red).

Fig. 4.
A model for the formation of nanotubes by IGFBP-2₄₉₋₂₈₉.