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Abstract. The development of adaptive nanomaterials that are responsive to changes in their surrounding environment would enable such materials to be used in wide range of applications such as drug delivery vehicles or biosensors. Reversible boronic ester chemistry, which is used in this work, has several advantages as a building block for making adaptive nanomaterials including the ease of preparation, high sensitivity to external stimuli such as pH, and relative stability especially when compared to other non-covalent reversible systems. Herein, by using small boronic acids as anchor and peptides as connectors, we report progress in the initial development of novel, peptidyl-based pH dependent adaptive nanomaterials using reversible boronic ester chemistry and its characterisation using small angle X-ray scattering.

1. Introduction

Peptide self-assembly has gained importance over the last few years amongst molecular self-assembled nanostructures [1, 2]. This is due to its unique properties involving modularity, biocompatibility, biodegradability, ease of synthesis and scalability. The ability to conjugate unnatural amino acids or creating hybrids by incorporating polymer, lipids and other materials in creating peptide-based self-assembled nanostructures is another advantage, giving extreme flexibility in the creation of functional materials. Similarly, peptide-based self-assembled materials offer advantages over proteins for the creation of biocompatible materials due to their far greater ease of synthesis, and typically greater stability - peptide-based building blocks are stable at extreme pH, high temperature, in the presence of organic solvents and mild to harsh conditions.

The self-assembly of peptides into higher-order structures or nanostructures is typically dependent on the combination of non-covalent interactions such as electrostatic interactions, hydrogen bonding, hydrophobic interactions and aromatic pi stacking between the building blocks. These non-covalent forces are weak when considered individually. However, the combination of forces leads to the self-organisation of simple building blocks into highly ordered nanostructures [2]. In this work we are using reversible covalent interactions, through boronic ester formation, as the principle structure-driving motif for these peptide structures, supplemented by the peptide self-organisation into secondary structure.

Nanostructures based on reversible boronic ester chemistry is a new arena where little research has been done so far [3, 4], but which shows potential for forming nanostructures with applications in areas

such as drug delivery due to their dynamic nature [5, 6]. Boronic acid or boronic ester linkage is a reversible system formed by covalent B-O bonds. These reversible covalent bonds can be used to create dynamic and adaptive complex systems similar to supramolecular, self-assembled systems [7], which are adaptive – responding to changes in external conditions with the composition of equilibrium re-equilibrates itself as the change occurs [8, 9]. Furthermore, these boronic ester-based systems act as pH stimuli-responsive systems and hence are being used for applications like drug delivery [10], biosensor [11] and neutron capture therapy [12].

Although covalent and reversible boronic ester linkages have been explored for some applications using DNA and polymers, it has not to our knowledge been used with peptides or proteins for forming nanostructures. Moreover, only phenylboronic acid has been known to conjugate with biomolecules for self-assembling nanostructures. The ability to tune and reverse self-assembled nanostructures into different shapes with more than one boronic acid in an aromatic system is unprecedented which is yet to be explored and studied. The resulting self-assembled nanostructures could potentially be useful in drug delivery, templating of other nanoparticles into 2D or 3D order based on the nature of the linkers used and the conditions.

There are various techniques used to study self-assembly of peptide or protein but small angle X-ray scattering (SAXS) is one of the few techniques that can study peptide self-assembly *in situ* in solution and track responses to environmental changes. Small angle scattering has been widely used to study the effect of protein templating on higher order structures and to characterise self-assembly of peptides in the solution state [13]. The technique provides information on the size, shape and structural orientation of self-assembled nanostructures [14-16]. It is also used to study reversible processes in their native conditions without the tedious preparation of samples. Challenges for SAXS in studying the self-assembly of biologically-based materials in water is the low scattering contrast between the materials and the surroundings, which make measurements at low concentrations difficult [14, 17]. It is also important to control the radiation time and experimental conditions to avoid radiation damage to the samples which may affect the observed structures (typically causing aggregation for biological samples) [14].

A key advantage of SAXS is that the sample is minimally perturbed during measurement. The use of other techniques like AFM, TEM has been shown to induce surface morphology of self-assembled systems due to either surface sample interaction or the sample preparation itself. For example, Saini *et al*. have shown that while characterizing with AFM and TEM, an octapeptide self-assemble to form fibers whereas in SAXS, it did not show peptide self-assembly at all [18]. Therefore, scattering becomes vital tool as a principal or complementary technique in confirming peptide or protein self-assembly. From SAXS we can not only derive structural information but the dynamicity of self-assembling processes as well. So, in this work, our aim is to explore characterising the novel, peptidyl pH adaptive nanomaterials developed through reversible ester chemistry using SAXS.

2. **Methodology**

2.1. Materials

1, 3, 5-phenyl tri-boronic acid was kindly provided by Seong Joo Nam of School of Chemical Sciences at the University of Auckland, synthesised and purified by using a known method [19].

A DOPA-modified neutral polyalanine peptide (Ac –DOPA-AAQAA AAQAA AAQAA-DOPA - NH_2, 1654.76 g/mol) was purchased from China Peptides, Shanghai, China. This peptide was synthesized by solid-phase method followed by purification using HPLC and mass analysis was performed with ESI-MS. The peptide was soluble up to 1mg/mL in water. The purity of these peptide was approximately 90%.

The phosphate buffer used for CD and SAXS experiments composition involves 30 mM of dibasic sodium phosphate and monobasic potassium phosphate at pH 7.4. All water used was ultrapure, with a resistivity of greater than 18 MΩ cm.

2.2. *Characterisation of peptide nanostructures using small angle X-ray scattering*
The final concentration used for peptide samples were set at 1 mg mL^{-1}. The ratio for 1, 3, 5-phenyl tri-boronic acid to peptide were kept at 1:100. The samples prepared using 30 mM phosphate buffer at pH 7.40. The samples used for reversibility experiments were prepared in ultrapure water. The pH was adjusted to acidic and basic conditions using 10 mM HCl and 10 mM NaOH.

The data collection was performed on Bruker SAXS instrument at ANSTO, Australia using a rotating anode Cu Kα source of X-rays (wavelength is 1.541 Å). The instrument contains 3 pinhole collimation and it has a detector made of VANTEC2000 2D detector with 68 μm resolution with 2048 by 2048 pixels. The Q range was set up from 0.01 $Å^{-1}$ to 0.4 $Å^{-1}$. A 1.5 mm quartz capillary tube was used to load the sample for the data collection. The buffers or the controls were run in all the capillary tubes before the data collection from the sample and the transmission of empty capillaries and glassy carbon were measured prior to the scattering measurements. The measurement time for samples in each capillary tubes were about 3 hours.

The data collected from the samples were then averaged and subtracted from buffers during the data reduction step. The transmission values from capillaries and glassy carbon were also accounted in the subtracted data. The data reduction was performed on Primus software of ATSAS suite [20, 21].

3. Results and Discussion

3.1. Selection of polyalanine peptide and its structure
In this study, a neutral polyalanine peptide modified with L- DOPA on both N and C-terminus to enable the formation of boronic ester chemistry. The peptide is neutral in charge and has 17 amino acid residues as shown in the *Figure 1*.The incorporation of L-DOPA at both ends of the peptide enables the formation of higher order structures, where the peptides form linkers between boronic acid anchors (joining points).

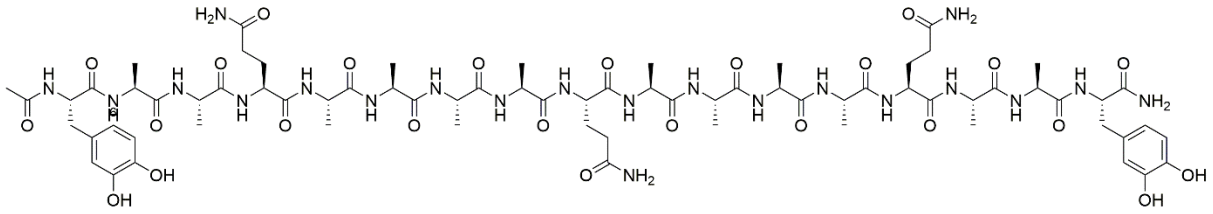

Figure 1: The structure of neutral polyalanine peptide with DOPA close to N and C terminus

The structure of the modified polyalanine peptide with L-DOPA was then characterized using Circular Dichroism spectroscopy to determine the secondary structure of the peptide in the conditions to be used for the material self-assembly. The CD spectra (*Figure 2)* shows that the neutral polyalanine peptide has a differential absorption spectrum characteristic of α-helical structure driven by internal hydrogen bonding. This implies that the peptides will form reasonably rigid linkers when incorporated into the self-assembled materials, with a predictable length and alignment of side-chain residues. Moreover, as the peptide is neutral this structure should be stable towards changes in pH and ionic strength of solution.

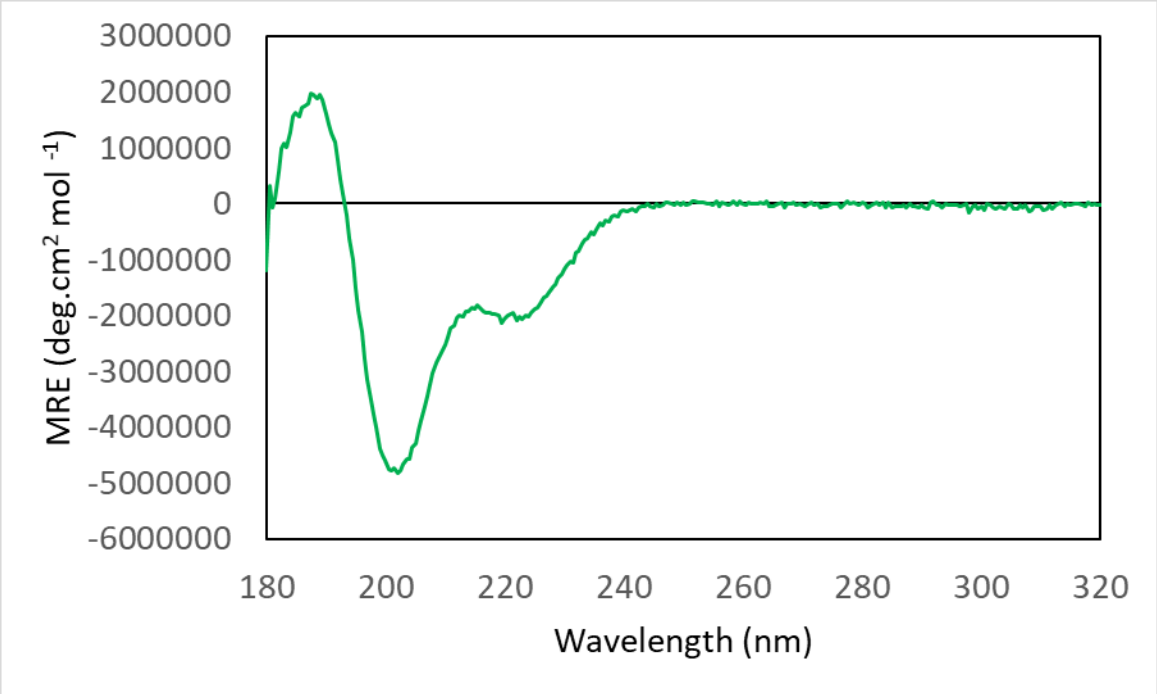

Figure 2: Circular dichroism (CD) spectra of the structure of neutral polyalanine peptide with DOPA

3.2. *Formation of peptide nanostructure characterised using small angle X-ray scattering*
Small angle X-ray scattering (SAXS) was performed on solutions of 1,3,5-phenyl tri-boronic acid (phTBA) and the peptide at a ratio of 1:100, which was previously optimised using NMR to show near complete formation of the borate ester in neutral conditions, as well as solutions of the peptide alone as a control to understand the unmediated peptide self-assembly. The concentration of the mixture was limited by the ultimate solubility of the phTBA to a maximum concentration of peptide of 1 mg/mL, which when combined with the low contrast between the components and water leads a relatively weak SAXS scattering pattern (*Figure 3*). Nevertheless it is clear that at neutral pH there is a significant difference between the complexes formed between the peptide and the phTBA and the peptide alone. There is a distinct increase in the scattering in the low-q scattering region ($q < 0.06$ Å^{-1}) for the peptide / phTBA combination at pH 7.4 than for the peptide alone. The scattering pattern did not change over the course of long term measurements (> 3 hours).

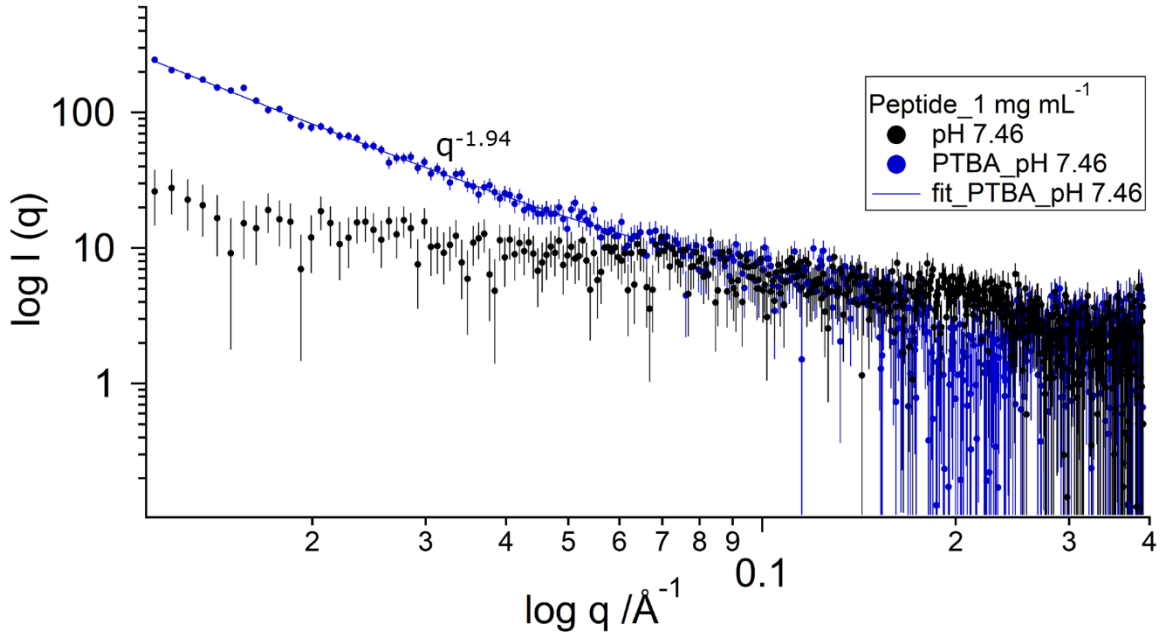

Figure 3: Experimental SAXS profile of peptide (black) and peptide-phTBA conjugates (blue) in solution. Upturn at low q region indicates aggregation or extended structures in the presence of phTBA at pH 7.46. The concentration of peptide used is 1 mg mL-1 in water and mixed in a ratio of 100:1 with 1, 3, 5 phenyl tri-boronic acid.

Table 1. The estimated parameters from the power-law curve fitting of the data in *Figure 5*

List of Parameters used for the power-law curve fitting	Estimated values and standard deviation
y_0	3.5 ± 2.7
A	0.039 ± 0.016
Power	-1.94 ± 0.09

This change in the low-q region indicates the formation of large scale structures. In this case the structures are larger than the measurement range of the SAXS camera used, which means that the structures formed are at least 100 nm in size, and the stability of the scattering pattern over time implies that these structure are not changing significantly over the course of hours. The power-law slope of this data is -1.94 ± 0.09, which indicates that the structure has mostly sheet characteristics. This is a clear indication that the peptides are bridging between phTBA anchors to form extended solution structures. Interestingly, although the peptide linkers are relatively rigid and the phTBA is in itself a planar structure, the structure formed is clearly more than a flat sheet. The flexibility of the linkages must lead to sufficient structural flexibility to enable the creation of these higher order structures. This suggest that the dimensionality can be adjusted by using different anchor points with a lower number of connection points (for example mono- or di-substituted phenyl boronic acids). The less substituted anchors should force the formation of structures with lower dimensionality (e.g., flat sheets, rods or chains), and combinations of substitutions of anchor may control of tailored options for the aggregate structure.

Although the resolution of this scattering is too poor to be definitive the scattering from the peptide-alone solution is not inconsistent with the scattering of isolated monomers. Although it is not possible

to be definitive on the shape of the monomers we can nevertheless confidently exclude any significant aggregation directly between peptide linkers in the absence of phTBA anchors.

In NMR studies (data not shown) we have shown that the hydrolysis of the boronate ester occurs under basic conditions (pH > 9). The samples that were measured in neutral conditions were raised in pH using dilute NaOH, and the SAXS scattering remeasured (*Figure 4*). In contrast to the samples measured at neutral pH there is no significant difference in these conditions between the peptides alone, and the peptide/phTBA complexes. This is further evidence that the higher order structures are a result of the formation of the covalent phTBA – peptide ester rather than through non-covalent interactions. When the pH is reduced again to neutral the higher-order structure reforms, indicating that the system is completely reversible.

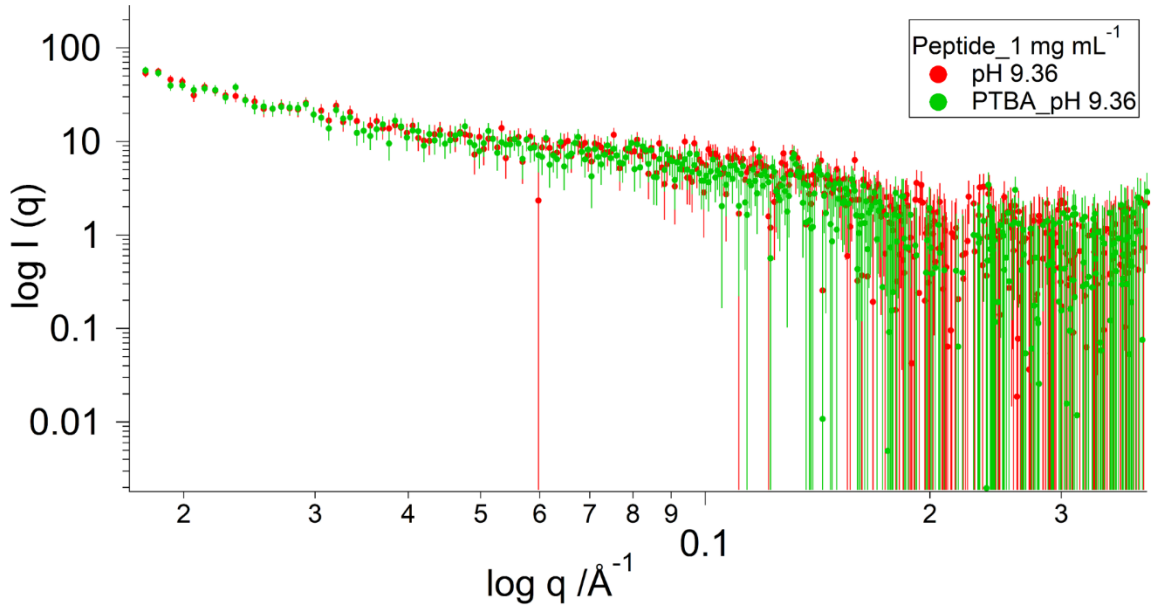

Figure 4: Experimental SAXS profile of peptide (red) and peptide-phenyl tri-boronic acid conjugates forming condition (green) in solution. No upturn at low q region indicates no sign of aggregation or extended structures in the presence of 1, 3, 5-phenyl tri-boronic acid at pH range of ___. The concentration of peptide used is 1 mg mL-1 in water and mixed in a ratio of 100:1 with 1, 3, 5-phenyl tri-boronic acid to form extended or possibly nanostructures.

The dependence of the higher-order structure on the pH of the environment, opens opportunities to develop materials that are responsive to changes in environmental conditions. The relatively small change in pH from around neutral to approximately 9 has resulted in complete loss of the higher order structure, and consequently the release of the peptides and anchors that were incorporated in the structure. As this system uses peptide-based linkers there is the additional possibility that this to be developed into a versatile delivery mechanism whereby molecules of interest can be connected to the material through tailored interactions with peptide sidechains, and exposed on change of pH.

4. Conclusion

In this work, we show the formation of higher order structures based on a boronic ester linkage motif using small peptides as linear linkers between 1, 3, 5-phenyl tri-boronic acid anchors (joining points) in aqueous solution at room temperature. The peptides by themselves show no propensity to self-associate,

but in the presence of the boronic acid formed large three-dimensional structures in solution at neutral pH of a size greater than 100 nm as shown through small angle X-ray scattering. The higher order structure is completely lost on increase of pH to release the linkers and anchor points, which we suggest may be used for a delivery system that is pH responsive. The system is biocompatible, and forms readily in ambient conditions.

The possibility of tailoring the higher-order structure that is formed by adjusting the anchor point (for example using mixtures of mono-, di- and tri- substituted phenylboronic acids to adjust the dimensionality and shapes formed) and the linker (for example using peptides with sidechains functionalised to bind to molecules of interest) mean that this system has high promise for the development of functional materials, which we will further explore.

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