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Incorporation of 'click' chemistry glycomimetics dramatically alters triple-helix stability in an adiponectin model peptide

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Adiponectin (Adpn) has been shown to be a possible therapeutic for Type II diabetes, however the production of a therapeutic version of Adpn has proved to be challenging. Biological studies have highlighted the importance of the glycosylated lysine residues for the formation of bioactive high molecular weight oligomers of Adpn. Through the use of 'click' glycopeptide mimetics, we investigated the role of glycosylated lysine and serine residues for the formation of triple helical structures of the collagenous domain of Adpn, in the context of a collagen model peptide scaffold. The physical properties of the unglycosylated lysine and serine peptides are compared with their glycosylated analogues. Our results highlight the crucial role of lysine residues for formation of the triple helical structure of Adpn, possibly due to the extension of both intra- and interstrand hydrogen bonding networks. Strikingly, we observed a significant decrease in thermal stability upon incorporation of triazole-linked analogues of glycosylated lysine residues into the adiponectin collageneous domain, indicating possible uses of 'click' glycomimetics for bioengineering applications.

Introduction

Adiponectin (Adpn) has been identified as playing a key role in the obesity-related diseases collectively known as 'metabolic syndrome'[1], including Type II diabetes mellitus (T2D),[2-4] cardiovascular disease[5, 6] and most recently melanoma;[7] these diseased states have all been shown to be associated with low levels of plasma adiponectin known as hypoadiponectinemia.[3,8] Expression of a therapeutic form of Adpn through recombinant techniques has proved challenging due to difficulties with purification of the Adpn oligomers produced in mammalian cell lines. Consequently, there has been significant interest in developing an understanding of the oligomerisation mechanism, in order to guide the chemical synthesis of a bioactive form of Adpn.

Human adiponectin is a 30 kDa peptide comprising of four segments, the *N*-terminal signal peptide (residues 1-21), the variable domain (residues 22-45), the collagenous domain (residues 46-107) and a *C*-terminal globular domain (residues 108-244) (Fig. 1). Adiponectin oligomerises *in vitro* to form higher-order structures, namely low molecular weight (LMW, *ca.* 180 kDa) and high molecular weight (HMW, *ca.* 400 kDa) oligomers[9-11]. The HMW oligomers of Adpn have been shown to be the most bioactive form of the protein[12]. Triple helix formation mediated by the C-terminal C1q-like globular domain, as well as the collagen-like domain, has been proposed as the first step in Adpn oligomerisation *in vivo*[13].

Notably, the collagenous domain of native Adpn lacks the characteristic 'GPO' Gly-Xaa-Yaa repeats that typically induce triple helix formation in collagen-type proteins. Indeed, we have previously demonstrated[14] that polypeptide constructs incorporating 30 residues of the N-terminal portion of the collagenous domain, in addition to the variable domain, are incapable of triple-helix formation *in vitro*.

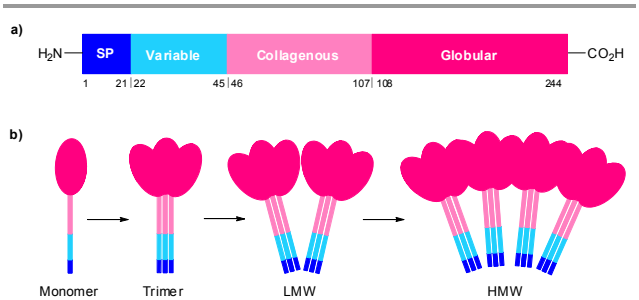

Fig. 1 a) The four segments of monomeric Adpn, and b) oligomers of Adpn.

We[15] and Richards *et al.*[16] have previously demonstrated the crucial role of post-translationally modified lysine (PTM-lysine) residues for formation of bioactive HMW

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oligomers of Adpn *in vivo*. For example, recombinant Adpn produced in *E. coli* cell lines are unable to form HMW oligomers, due to the lack of PTMs in bacterially produced proteins[17]. In 1967, Spiro *et al.*[18] elucidated the chemical structure of the PTM-lysine residues found in Adpn to be the disaccharide unit 2-*O*-α-D-glucopyranosyl-D-galactose bound to (2*S*,5*R*)-hydroxy-lysine by a β-glycosidic bond, namely Glu-Gal-Hyl **1** (Fig. 2).

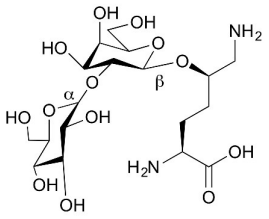

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Fig. 2 The chemical structure of PTM-lysine residues found in Adpn namely Glu-Gal-Hyl **1**.

In 2016 Ohkubo *et al.*[19] demonstrated that synthetic Adpn peptides derived from the collagenous domain containing these PTM-lysine residues are able to form triple helical secondary structures in solution. In addition, adoption of the triple helical structures of Adpn peptides was shown to be the crucial first step for oligomerisation of the peptides, with spontaneous self-assembly into an octodecamer occurring at physiological pH at 20 °C. This highlights the importance of the PTM-lysine residues for the formation of bioactive HMW oligomers of Adpn.

At the outset of this study, we sought a minimalist (glyco)peptide model system that would recapitulate the triple helix formation observed in native adiponectin, with the aim of establishing a facile model system for the investigation of chemical modulation of this structure by post-translational modification. Collagen model peptides (CMPs) have been shown to be a good mimetic for collagenous peptides[20,14, 21]. Our group has previously demonstrated[14] that incorporation of a 'guest' peptide sequence derived from Adpn(residues 18-74) into a classical GPO collagen repeat 'host' sequence 'rescues' the disfavoured triple-helix formation in such constructs. Thus, in the present study a CMP 'host' $(GPO)_3$ construct was appended on either side of the 18-mer 'guest' Adpn domain as a mimic for the remainder of the Adpn peptide (Fig. 3).

To circumvent the synthetic difficulties associated with the preparation of native glycopeptide constructs, various approaches informed by the 'click chemistry'[22] concept have been reported to provide expedient access to non-natural, but biologically relevant glycoconjugates (termed by us neoglycosides/ neoglycopeptides)[23-29]. Such glycoconjugates are particularly straightforward to prepare using the Cu(I)-catalysed azide-alkyne cycloaddition 'click' (CuAAC) reaction. The CuAAC reaction is neither air nor moisture sensitive, and is high yielding and flexible in terms of solvent and protecting group choice[30]. Importantly, such *neo*glycopeptides have been shown to be more resistant to digestive enzymes, thus making the triazole moiety an attractive linker for potential biopharmaceuticals[31, 32].

We therefore decided to investigate whether 'click' analogues of PTM-lysine could modulate the triple helical propensity in a peptide construct derived from the Adpn collagenous domain. As a proof-of-principle, we selected monosaccharide analogues of native PTM-lysine **1**, 'click' galactolysine **2** and 'click' galactoserine **3** (Fig. 3) in order to identify the minimal structure required to induce triple helical propensity in Adpn glycopeptides. We rationalised this structural simplification as, while native Adpn contains the unusual PTM Glu-Gal-Hyl **1**, structurally simpler glycosylated residues have also been demonstrated to impart triple helical propensity to collagen-type polypeptides[33-36]. Notably, β-galactosylated threonine is found in the place of hydroxyproline in collagens from the deep-sea hydrothermal vent worm *Riftia pachyptila*, where the glycosylated residue is essential for triple helix formation[33, 34]. Further, studies from the Wennemers group[20, 37] have demonstrated the tolerance of triple-helical structures for triazole-linked monosaccharides in the Yaa position of CMP Gly-Xaa-Yaa repeats.

We chose to incorporate 'click' glycosides into an 18-amino acid segment of the collagenous domain of Adpn (residues 66-83), as three of the four PTM-lysine residues occur within this segment in native Adpn[15]. Serine 'click' glycosides were synthesised alongside the lysine glycosides to probe the importance of the interspace distance between the glycan and the peptide backbone. In a similar manner to Ohkubo *et al.*,[19] the structural triple helical properties of our resultant 36-mer CMP-Adpn peptides **4**-**7** were then investigated using circular dichroism.

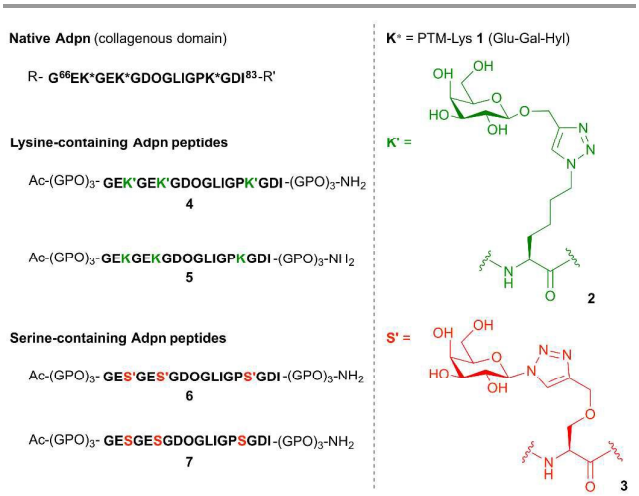

Fig. 3 Family of CMP-Adpn peptides **4-7** prepared in this study. The Adpn 'guest' sequence is highlighted in **bold**.

We envisaged that the CMP-Adpn peptides **4**-**7** could be prepared by Fmoc-SPPS, and the *neo*glycosides in glycopeptides **4** and **6** could be incorporated as the building

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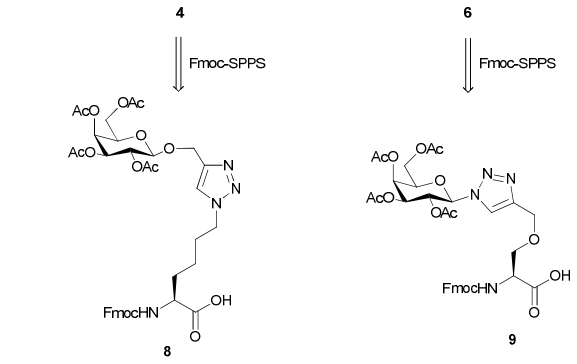
blocks Fmoc-Lys(*neo*Gal(OAc))-OH **8** and Fmoc-Ser(*neo*Gal(OAc))-OH **9**, respectively (Scheme 1). Acetate protecting groups were chosen for the glycan hydroxyl groups as these could be easily removed using the Zemplén[38] procedure following peptide synthesis.



Scheme 1 Retrosynthetic strategy for *neo*glycopeptides **4** and **6**.

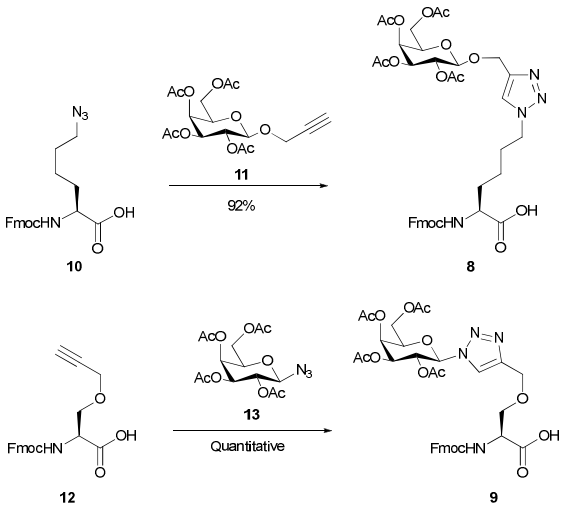
Results and discussion

Synthesis

For the preparation of Fmoc-Lys(*neo*Gal(OAc))-OH **8** and Fmoc-Ser(*neo*Gal(OAc))-OH **9**, the required pre-'click' building blocks namely Fmoc-lysine-azide **10**[39], 1-*O*-propargyl-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranose **11**[40], Fmoc-*O*-propargyl-serine **12**,[40] and 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl azide **13**[42] were first synthesised following literature methods. The pre-'click' coupling partners **10** and **11**, and **12** and **13**, were then subjected to the CuAAC 'click' reaction using copper(II) sulfate and sodium ascorbate[41] with heating at 80 °C under microwave irradiation for 1 h (Scheme 2). The resulting Fmoc-protected triazole-linked glycoside products **8** and **9** were isolated in 92% and quantitative yield respectively, ready for incorporation into Adpn peptides **4** and **6**. Both **8** and **9** were found to be stable at room temperature and soluble in a range of organic and protic solvents.

All four CMP-Adpn peptides **4-7** were subsequently prepared by Fmoc-SPPS on an aminomethyl polystyrene resin functionalised with a Rink-amide linker **14** (Scheme 3, see Supporting Information for details).

Briefly, the Fmoc-protecting groups were removed with a solution of piperidine in DMF and condensation of each subsequent amino acid residue was performed with either HATU or HCTU, either at room temperature or with microwave irradiation. Upon completion of the peptide sequence, the *N*-terminal residue of the resin bound peptides was *N*-acetylated with acetic anhydride and the acetylated peptides released from the resin using a cleavage cocktail of TFA/TIPS/H_2O. Subsequent removal of the acetate protecting groups on the carbohydrate residues, followed by purification using reverse phase high performance liquid chromatography (RP-HPLC) afforded the CMP-Adpn peptides **4**-**7** in good purity (see Supporting Information).



Scheme 2 Synthesis of *neo*galactolysine **8** and *neo*galactoserine **9** using the CuAAC 'click' reaction. *Reagents and conditions:* Sugar donor **11** or **13** (1.1 eq), $CuSO_4{\cdot}5H_2O$ (0.3 eq), sodium ascorbate (0.6 eq), degassed EtOH, μw, 80 °C, 1 h.

Circular dichroism studies

We next investigated the triple helical properties of CMP-Adpn peptides **4**-**7** using circular dichroism (CD), where a triple helix is signified by a maximum at 225 nm and a minimum at 198 nm[42]. Peptides **4**-**7** were dissolved in a potassium phosphate buffer solution at pH 7.4 and the CD spectra obtained at 6 °C (Fig. 4). The CD profiles revealed that the lysine-containing Adpn peptides **4** and **5** displayed signature triple helical features and were thus able to form a triple helix. In contrast, the serine-containing Adpn peptides **6** and **7** were unable to adopt this secondary structure, instead adopting a random-coil conformation. In addition, the similarity of the CD profiles for lysine-containing peptides **4** and **5**, and serine-containing peptides **6** and **7** indicated that the type of residue (lysine or serine) had a larger effect on triple helical conformation than the 'clicked' glycan moieties.

For lysine-containing Adpn peptides **4** and **5**, thermal melt studies were conducted at 215 nm to investigate the thermal stabilities of these peptides. The triazole-linked 'click' glycosylated peptide **4** was found to have a significantly lower melting point (T_m of 20.4 °C) compared to the non-glycosylated peptide **5** (T_m of 40.9 °C) (Fig. 5). This indicated that 'click'

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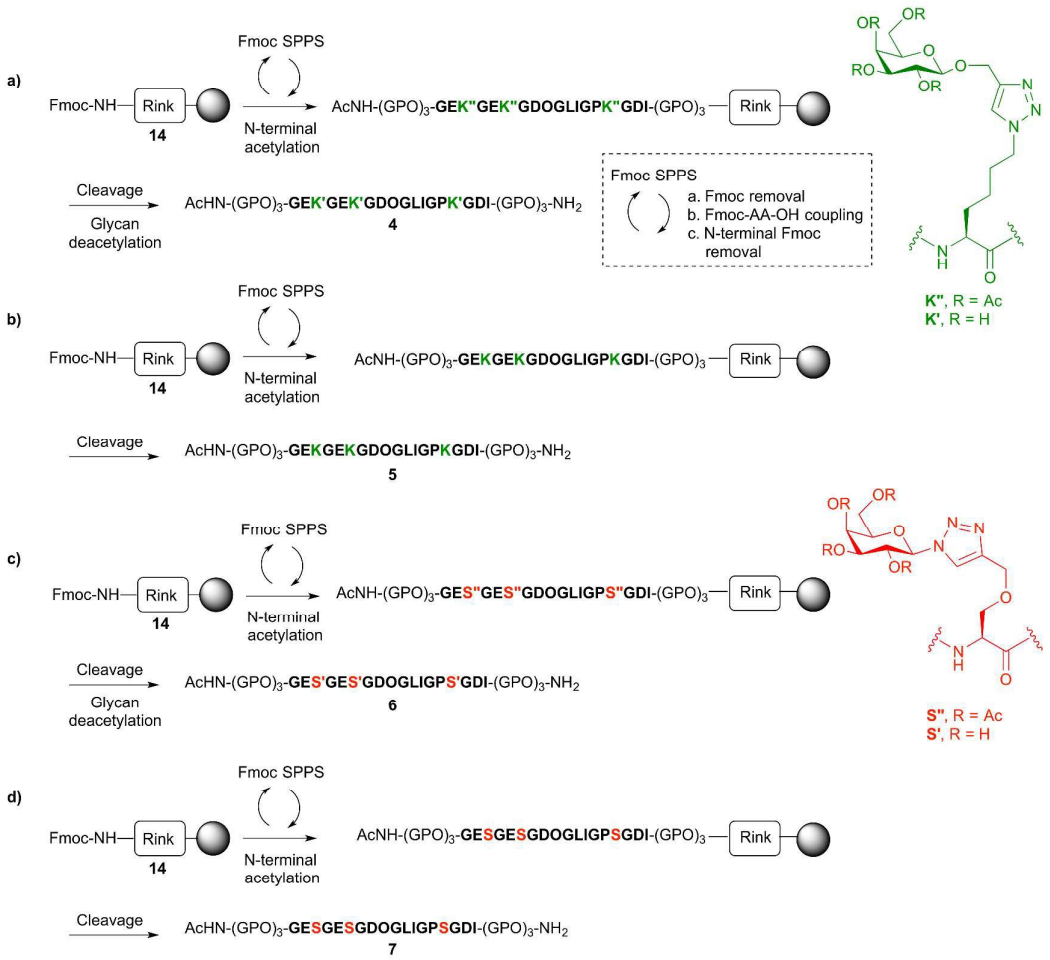

Scheme 3 Synthesis of (a) **4**, (b) **5**, (c) **6** and (d) **7**. Adpn-derived sequences are highlighted in **bold**. See Supplementary Information for full experimental details.

Reagents and conditions: (a) **4**: Fmoc removal: 20% piperidine-DMF, 10 min, r.t. **or** 40% piperidine-DMF, µW, 75 °C, 25 W, 6 min; Fmoc-AA-OH coupling: Fmoc-amino acid (5 eq), HCTU (4.6 eq), DIPEA (10 eq), NMP/DMF (1:4), 45 min, r.t. **or** Fmoc-amino acid (5 eq), HCTU (4.5 eq), DIPEA (10 eq), NMP/DMF (1:5), µW, 75 °C, 25 W, 5 min ; Coupling of glyco-AA **8**: **8** (5 eq), HATU (4.6 eq), DIPEA (10 eq), DMF, µW, 75 °C, 25 W, 5 min; N-terminal acetylation: Ac_2O (40 eq), DIPEA (10 eq), NMP, 2 x 20 min, r.t.; Resin cleavage: TFA/TIPS/H_2O (93:3.5:3.5), µW, 35 °C, 10 W, 20 min; Glycan deacetylation: NaOMe/MeOH (1 M), 2 h, r.t.

(b) **5**: Fmoc removal: 20% piperidine-DMF, 10 min, r.t.; Fmoc-AA-OH coupling: Fmoc-amino acid (5 eq), HCTU (4.6 eq), DIPEA (10 eq), NMP/DMF (1:4), 45 min, r.t.; N-terminal acetylation: Ac_2O (40 eq), DIPEA (10 eq), NMP, 2 x 20 min, r.t.; Resin cleavage: TFA/TIPS/H_2O (90:5:5), 90 min, r.t.

(c) **6**: Fmoc removal: 20% piperidine-DMF, 10 min, r.t. **or** 20% piperidine-DMF, µW, 75 °C, 25 W, 6 min; Fmoc-AA-OH coupling: Fmoc-amino acid (5 eq), HCTU (4.6 eq), DIPEA (10 eq), NMP/DMF (1:4), 45 min, r.t. **or** Fmoc-amino acid (5 eq), HATU (4.6 eq), DIPEA (10 eq), DMF, µW, 75 °C, 25 W, 5 min **or** Fmoc-amino acid (10 eq), HCTU (7 eq), NMM (10 eq), NMP/DMF (1:4), 20 min, r.t. N-terminal acetylation: Ac_2O (40 eq), DIPEA (10 eq), NMP, 2 x 20 min, r.t.; Resin cleavage: TFA/TIPS/H_2O (90:5:5), 90 min, r.t.; Glycan deacetylation: NaOMe/MeOH (1 M), 2 h, r.t.

(d) **7**: Fmoc removal: 20% piperidine-DMF, 10 min, r.t.; Fmoc-AA-OH coupling: Fmoc-amino acid (5 eq), HCTU (4.6 eq), DIPEA (10 eq), NMP/DMF (1:4), 45 min, r.t.; N-terminal acetylation: Ac_2O (40 eq), DIPEA (10 eq), NMP, 2 x 20 min, r.t.; Resin cleavage: TFA/TIPS/H_2O (90:5:5), 90 min, r.t.

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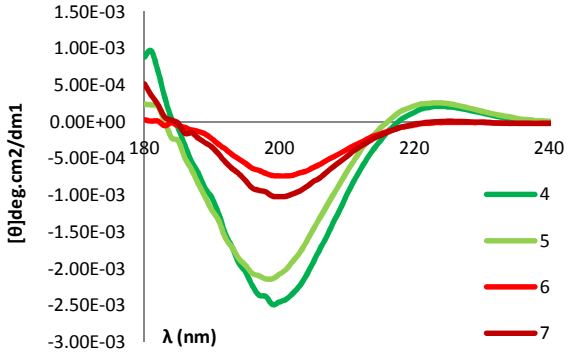

Fig. 4 CD spectra of the CMP-Adpn peptides **4**-**7**.

glycosylation of the lysine residue had a significant destabilising effect on the triple helical structure.

Discussion

The ability of collagen-like peptides to form a triple helix is dependent upon a favourable combination of steric constraints, interstrand hydrogen bonding networks and electrostatic interactions[43, 44]. For glycosylated CMPs the hydrogen bonding network extends to additional glycan-glycan and glycan-peptide backbone interactions[45].

The difference in triple helix formation observed for the lysine- and serine-containing CMP-Adpn peptides highlights the critical role of lysine residues in the Yaa position for triple helix induction in Adpn peptides. The importance of lysine in this position could be due to both hydrogen bonding and electrostatic interactions, dependent upon the length of the amino acid side-chain. We postulate that the longer lysine side-chain length in lysine-containing Adpn peptides **4** and **5** facilitates the formation of intrastrand and interstrand hydrogen bonding networks, thus stabilising the triple helical structure. Conversely, the serine analogues **6** and **7** may not be able to adopt the correct conformation for hydrogen bonding, thus destabilising the structure and resulting in a random coil conformation.

In addition to favourable hydrogen bonding interactions, the lysine-containing Adpn peptide **4** may also have additional electrostatic interactions favouring the collagen-type triple helical structure, which the serine analogues **6** and **7** may lack. Several groups[46-49] have reported the importance of electrostatic interactions between positively and negatively charged residues for the structural stability of collagens. Indeed, Brodsky *et al.*[46] noted that ionisable residues (Lys, Arg, Glu and Asp) account for 15-20% of total residues in collagenous peptides, indicating a key role for such electrostatic interactions in the formation and/or stabilisation of the triple helical structure and subsequent fibril formation of collagenous peptides.

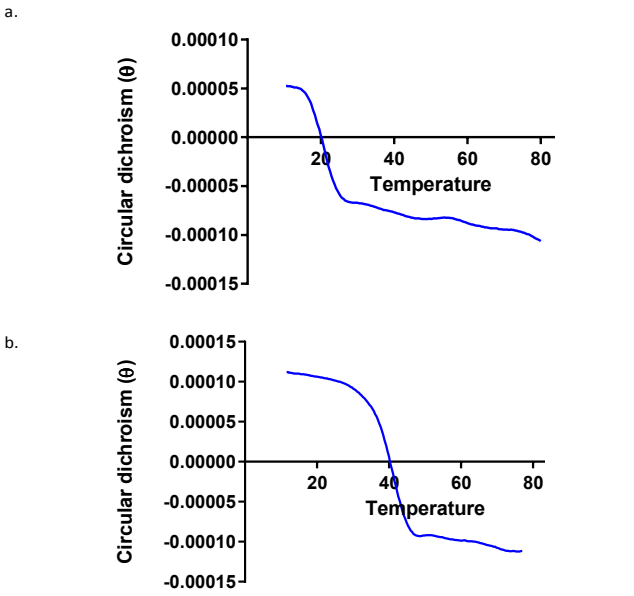

Fig. 5 Thermal melt curves for a) 'click' *neo*glycosylated Adpn peptide **4** and b) unglycosylated-Adpn peptide **5**.

In the case of non-glycosylated peptide **5** it is probable that, in the tested buffer solution at pH 7.4, the ionised Lys residues may form stabilising ion-pairs with neighbouring strand Glu residues, thus stabilising the triple helical structure of the peptide. Furthermore, the charged NH_3^+ residues present in **5** could also have contributed to a stabilising hydrogen bonding network, similar to those observed in published crystal structures of CMPs such as H-$(POG)_4$EKG$(POG)_5$-OH reported by Berman *et al.*[50]

It is interesting to note that incorporation of triazole-linked β-galactose into the lysine-containing Adpn peptide **5** dramatically lowered the thermal stability of glycosylated peptide **4**. This is in stark contrast to previous studies of glycosylated CMP scaffolds in which glycosylation has typically resulted in either enhanced triple helical propensity and thermal stability, or only moderate reduction. Most dramatically, Bann et al.[34] synthesised repeat sequences of the (Gly-Pro-Thr) motif found in the cuticle collagen of the hydrothermal vent worm *Riftia pachyptila* and demonstrated triple helix formation only if the threonine residue was β-galactosylated. The stabilising influence of galactosylation in that system was ascribed to carbohydrate-mediated 'shielding' of peptide amide-water hydrogen bonds, favouring the formation of inter-strand hydrogen bonding and thus triple helix formation. Conformational restriction of the galactosyl residue due to the presence of the triazole linker may prevent such a mechanism operating in the case of our glycosylated peptide **4**. Further, linker length has been shown to be a key variable in the conformational preferences of glycopeptides[51], an observation consistent with the differing triple helical propensities in 'click' glycopeptides **4** and **6**.

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Wennemers *et al.*[20] have reported functionalization of azidoproline-containing CMP constructs with galactosyl residues. In these studies, only a relatively slight decrease (5 °C) in thermal stability was reported upon incorporation of a triazole-linked galactosylated hydroxyproline (O*) residue into the CMP Ac-$(POG)_3$PO*G-$(POG)_3$-NH_2.

The observed decrease in thermal stability is also in contrast to previous literature reports of native glycosylated Adpn[33] and the recently published work by Ohkubo *et al.* [19] where a native glycosylated peptide of the collagenous domain of Adpn (residues 19-107) was found to be more thermally stable (T_m of 33.4 °C) than the unglycosylated peptide analogue (T_m of 12.1 °C). This contrast may point to additional glycan-dependent interactions not recapitulated by incorporation of shorter monosaccharides. While insertion of the Adpn sequence into CMP host sequences is here shown to stabilise triple helical structures, it is likely that the specific conformation is altered relative to the native protein. Indeed, CMPs have been shown to adopt a tighter 7/2 triple helix[52] in contrast to collagenous peptides which favour a looser 10/3 conformation[53]. The tighter helix could alter steric constraints, glycan-glycan interstrand and glycan-peptide backbone interactions, thus altering the thermal stability observed for peptide **4**.

This significant difference in thermal stability upon incorporation of triazole-linked *neo*glycans into Adpn peptides compared to native glycosylated Adpn peptides[19] provides a significant opportunity for the use of Adpn 'guest' sequences in concert with 'click' *neo*glycosides in collagen-type materials to manipulate the thermal properties of these materials. Raines *et al.*[54] have already noted the use of collagen-based materials for wound healing and nanowire applications. Our findings indicate that incorporation of *neo*glycosides, perhaps via a 'click and switch' mechanism, could be useful for altering the thermal stabilities of such materials.

Conclusions

Our synthetic studies and subsequent structural investigations have demonstrated the structural importance of lysine and glycosylated lysine residues for formation of a triple helical secondary structure for a section of the collagenous domain of Adpn. Intriguingly, only the lysine-containing peptides, in both the native and *neo*glycosylated state, were able to adopt the desired triple helical structure with the serine-containing analogues adopting a random coil conformation instead. This result highlights the importance of side-chain length for probable hydrogen bonding and electrostatic contributions to the triple helical secondary structure. Furthermore, comparison of the thermal stabilities of 'click' glycopeptide **4** and non-glycosylated **5** demonstrated that incorporation of triazole-linked *neo*glycosides into the adiponectin sequence lowered the thermal stability of these peptides. Our findings could have key implications for the use of such 'click' glycosides to modify the thermal properties of collagenous materials.

Acknowledgements

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References

1. I. Padmalayam and M. Suto, *Current pharmaceutical design*, 2013, **19**, 5755-5763.
2. K. Hara, P. Boutin, Y. Mori, K. Tobe, C. Dina, K. Yasuda, T. Yamauchi, S. Otabe, T. Okada and K. Eto, *Diabetes*, 2002, **51**, 536-540.
3. G. Valsamakis, R. Chetty, P. G. McTernan, N. M. Al-Daghri, A. Barnett and S. Kumar, *Diabetes, Obesity and Metabolism*, 2003, **5**, 131-135.
4. M. Goto, A. Goto, A. Morita, K. Deura, S. Sasaki, N. Aiba, T. Shimbo, Y. Terauchi, M. Miyachi and M. Noda, *Obesity*, 2014, **22**, 401-407.
5. T. Maia-Fernandes, R. Roncon-Albuquerque Jr and A. Leite-Moreira, *Revista portuguesa de cardiologia: orgao oficial da Sociedade Portuguesa de Cardiologia= Portuguese journal of cardiology: an official journal of the Portuguese Society of Cardiology*, 2008, **27**, 1431-1449.
6. Y. Okamoto, S. Kihara, N. Ouchi, M. Nishida, Y. Arita, M. Kumada, K. Ohashi, N. Sakai, I. Shimomura and H. Kobayashi, *Circulation*, 2002, **106**, 2767-2770.
7. Y. Sun and H. F. Lodish, *PLoS One*, 2010, **5**, e11987.
8. K. Maeda, K. Okubo, I. Shimomura, T. Funahashi, Y. Matsuzawa and K. Matsubara, *Biochemical and biophysical research communications*, 1996, **221**, 286-289.
9. H. Ruotsalainen, M. Risteli, C. Wang, Y. Wang, M. Karppinen, U. Bergmann, A.-P. Kvist, H. Pospiech, K.-H. Herzig and R. Myllylä, *PloS one*, 2012, **7**, e50045.
10. M. Trujillo and P. Scherer, *Journal of internal medicine*, 2005, **257**, 167-175.
11. T.-S. Tsao, E. Tomas, H. E. Murrey, C. Hug, D. H. Lee, N. B. Ruderman, J. E. Heuser and H. F. Lodish, *Journal of Biological Chemistry*, 2003, **278**, 50810-50817.
12. A. H. Berg, T. P. Combs, X. Du, M. Brownlee and P. E. Scherer, *Nature medicine*, 2001, **7**, 947.
13. T.-S. Tsao, *Reviews in Endocrine and Metabolic Disorders*, 2014, **15**, 125-136.
14. P. W. Harris, L. Hampe, M. Radjainia, M. A. Brimble and A. K. Mitra, *Peptide Science*, 2014, **102**, 313-321.
15. Y. Wang, K. S. Lam, L. Chan, K. W. Chan, J. B. Lam, M. C. Lam, R. C. Hoo, W. W. Mak, G. J. Cooper and A. Xu, *Journal of Biological Chemistry*, 2006, **281**, 16391-16400.
16. A. A. Richards, T. Stephens, H. K. Charlton, A. Jones, G. A. Macdonald, J. B. Prins and J. P. Whitehead, *Molecular endocrinology*, 2006, **20**, 1673-1687.
17. Y. Wang, A. Xu, C. Knight, L. Y. Xu and G. J. Cooper, *Journal of Biological Chemistry*, 2002, **277**, 19521-19529.
18. R. G. Spiro, *Journal of Biological Chemistry*, 1967, **242**, 4813-4823.
19. A. Takuwa, T. Yoshida, T. Maruno, K. Kawahara, M. Mochizuki, Y. Nishiuchi, Y. Kobayashi, T. Ohkubo, Y. Hoshi and K. Endo, *FEBS Lett*, 2016, **590**, 195-201.

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20. R. S. Erdmann and H. Wennemers, *Organic & biomolecular chemistry*, 2012, **10**, 1982-1986.
21. G. B. Fields, *Organic & biomolecular chemistry*, 2010, **8**, 1237-1258.
22. H. C. Kolb, M. Finn and K. B. Sharpless, *Angewandte Chemie International Edition*, 2001, **40**, 2004-2021.
23. N. Miller, G. M. Williams and M. A. Brimble, *Organic letters*, 2009, **11**, 2409-2412.
24. D. J. Lee, K. Mandal, P. W. Harris, M. A. Brimble and S. B. Kent, *Organic letters*, 2009, **11**, 5270-5273.
25. R. Peltier, M. A. Brimble, J. M. Wojnar, D. E. Williams, C. W. Evans and A. L. DeVries, *Chemical Science*, 2010, **1**, 538-551.
26. D. J. Lee, P. W. Harris and M. A. Brimble, *Organic & biomolecular chemistry*, 2011, **9**, 1621-1626.
27. M. A. Brimble, N. Miller and G. M. Williams, *Amino Acids, Peptides and Proteins in Organic Chemistry: Protection Reactions, Medicinal Chemistry, Combinatorial Synthesis, Volume 4*, 2011, 359-391.
28. D. J. Lee, S.-H. Yang, G. M. Williams and M. A. Brimble, *The Journal of organic chemistry*, 2012, **77**, 7564-7571.
29. J. M. Wojnar, D. J. Lee, C. W. Evans, K. Mandal, S. B. Kent and M. A. Brimble, *Click Chemistry in Glycoscience: New Developments and Strategies*, 2013, 251-270.
30. J. Thundimadathil, *Chim. Oggi-Chem. Today*, 2013, **31**, 34-37.
31. I. E. Valverde, A. Bauman, C. A. Kluba, S. Vomstein, M. A. Walter and T. L. Mindt, *Angewandte Chemie International Edition*, 2013, **52**, 8957-8960.
32. J. M. Wojnar, C. W. Evans, A. L. DeVries and M. A. Brimble, *Australian Journal of Chemistry*, 2011, **64**, 723-731.
33. J. G. Bann, D. H. Peyton and H. P. Bächinger, *FEBS letters*, 2000, **473**, 237-240.
34. J. G. Bann, H. P. Bächinger and D. H. Peyton, *Biochemistry*, 2003, **42**, 4042-4048.
35. N. W. Owens, J. r. Stetefeld, E. Lattová and F. Schweizer, *Journal of the American Chemical Society*, 2010, **132**, 5036-5042.
36. P.-W. Huang, J.-M. Chang and J.-C. Horng, *Amino acids*, 2016, **48**, 2765-2772.
37. R. S. Erdmann and H. Wennemers, *Journal of the American Chemical Society*, 2010, **132**, 13957-13959.
38. G. Zemplén and A. Kunz, *European Journal of Inorganic Chemistry*, 1924, **57**, 1357-1359.
39. T. J. Sminia and D. S. Pedersen, *Synlett*, 2012, **23**, 2643-2646.
40. S. Muthana, H. Yu, S. Huang and X. Chen, *Journal of the American Chemical Society*, 2007, **129**, 11918-11919.
41. T. H. Wright, A. E. Brooks, A. J. Didsbury, J. D. McIntosh, K. Burkert, H. Yeung, G. M. Williams, P. R. Dunbar and M. A. Brimble, *Synlett*, 2013, **24**, 1835-1841.
42. A. S. Parmar, A. M. Nunes, J. Baum and B. Brodsky, *Biopolymers*, 2012, **97**, 795-806.
43. K. Kar, P. Amin, M. A. Bryan, A. V. Persikov, A. Mohs, Y.-H. Wang and B. Brodsky, *Journal of Biological Chemistry*, 2006, **281**, 33283-33290.
44. M. D. Shoulders and R. T. Raines, *Annual review of biochemistry*, 2009, **78**, 929-958.
45. E. B. Naziga, F. Schweizer and S. D. Wetmore, *The Journal of Physical Chemistry B*, 2013, **117**, 2671-2681.
46. M. G. Venugopal, J. A. Ramshaw, E. Braswell, D. Zhu and B. Brodsky, *Biochemistry*, 1994, **33**, 7948-7956.
47. J. A. Fallas, J. Dong, Y. J. Tao and J. D. Hartgerink, *Journal of Biological Chemistry*, 2012, **287**, 8039-8047.
48. S. Kumar and R. Nussinov, *ChemBioChem*, 2002, **3**, 604-617.
49. T. Gurry, P. S. Nerenberg and C. M. Stultz, *Biophysical journal*, 2010, **98**, 2634-2643.
50. R. Z. Kramer, M. G. Venugopal, J. Bella, P. Mayville, B. Brodsky and H. M. Berman, *Journal of molecular biology*, 2000, **301**, 1191-1205.
51. Y. K. Sunkari, F. Alam, P. S. Kandiyal, S. Aloysius, R. S. Ampapathi and T. K. Chakraborty, *ChemBioChem*, 2016, **17**, 1839-1844.
52. R. Z. Kramer, J. Bella, P. Mayville, B. Brodsky and H. M. Berman, *Nature structural biology*, 1999, **6**, 454-457.
53. J. Bella, J. Liu, R. Kramer, B. Brodsky and H. M. Berman, *Journal of molecular biology*, 2006, **362**, 298-311.
54. S. Chattopadhyay and R. T. Raines, *Biopolymers*, 2014, **101**, 821-833.