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Journal:	*Chemical Science*
Manuscript ID:	Draft
Article Type:	Edge Article
Date Submitted by the Author:	n/a

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Cite this: DOI: 10.1039/c0xx00000x

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EDGE ARTICLE

Collagen neoglycosylation at lysine residues: design synthesis and characterization

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Collagen scaffolds have been glycosylated with lactose by reductive amination at lysine side chains. AFM analysis highlights that the chemical glycosylation does not affect molecular assembly into fibrils, and increases surface wettability compared to the untreated sample, as expected by theoretical calculation. Moreover, ELLA biochemical assays show that galactose unit of lactose is efficiently exposed on the scaffold surface for recognition by its receptor and, more intriguing from a chemical perspective, for inclusion of different functional saccharidic epitopes.

Introduction

The design of new protein-based macromolecules has mushroomed over the past decade. Works in this field present both challenges in base science such as understanding of biomolecular assembly mechanisms, and opportunities in material science, bionanotechnology, and for applications in tissue engineering. At present there is a pressing need to selectively modify peptides to produce a self-standing functional system.[1] These systems can be very challenging to prepare due to the high level of functionality and of structural complexity of the protein themselves. In this regard challenges include avoiding non-specific side chain reactions, and assuring a proper function through orthogonal modification. These are key aspects when advanced properties (i.e. catalytic, recognition, mechanic, signalling) must be preserved. Because of the higher accessibility surface, linear or fibrous proteins offer a prised solution with respect to globular proteins: the outer exposition of the amino acid side chains observed in the first class of protein is unviable for the other class. One of the main representative of the first family is collagen. Collagen molecule has a triple-helix structure[2] formed by the assembly of three polypeptide chains each in a polyproline II-like conformation and supercoiled around a common axis. The three chains are staggered by one residue and their close packing near this axis requires every third amino acid to be glycine (G) thus generating the characteristic G-X-Y repeating pattern observed in collagen. Among collagens, collagen type I represents the main molecular building block of all the connective tissues involved in structural functions. Both its primary sequence (NP_000079,a1(I); NP_000080, a2(I)) and its conformation are known thus allowing to assess the position of a given residue type along the molecule and hence to identify the position and the accessibility of feasible binding sites along the collagen molecule. In the case of type I collagen the triple-helix structure is stabilized by its high content of Proline (P) and hydoxyproline (O) which promote PPII helix stability. The triple helix structure of collagen allows short peptide sequences to be used for the study of collagen stability both experimentally and theoretically. Accordingly the high propensity observed for the triplets G-P-O reflected on the sequences studied in the literature so far.

In recent years, the development of collagen based functional materials has gained a lot of interest.[3] Such materials are useful as scaffolds for cell growth, for wound healing and the development of artificial skin. The design of collagen scaffolds that can be easily functionalized with signalling biomolecules in order to upgrade collagen to a cell-responsive scaffold, without altering its structural features is of primary importance. Despite their noted functional role,[4] glycans have had limited use in biomaterial design.[5] In additions, in recent years, collagen glycosylation is emerging as a key issue in the control of cartilage formation, growth, metabolism, and repair.[6]

In the present work we explore whether collagen-based 2D scaffolds may be functionalized with glycan moieties without affecting its structural features. In this respect, theoretical and molecular modelling are valuable tools to guide the design of collagen-based materials.[7] Performing residue-specific chemical reactions on collagen and maintaining its integrity is not an easy task.[8] Different strategies have been proposed during the years for protein bioconjugation;[9] most of them usually target functionalities present in the side chains of the canonical, proteogenic amino acids, where cysteine[10] and lysine[11] are the most exploited residues.

In the proposed work, a bioconjugation approach targeting lysine

residues was chosen for the linkage with a disaccharide such as lactose. The choice of a simple disaccharide was driven by the fact that collagen is reported to be glycosylated with small glycidic epitopes, that do not possess more than 2 monosaccharidic units. Lysines bear a primary amine mojety reactions that have proven useful for bioconjugation is the coupling of the amino group with carbonyls, affording the corresponding Schiff base or a secondary amine after imine reduction[12].

Result and Discussion

Molecular Design

Molecular modeling of lysine-specific modification of collagen

Molecular dynamics simulations are performed to assess the equilibrium configuration of the wild-type and functionalized collagen molecule (see Figure 1). The results show that the wild-type lysine is exposed towards the solvent although it lies close to the triple helix. On the other hand, the lactose functionalization is well exposed and particularly the glucose ring is highly solvated. Partial charge calculations show that lysine side chain presents neutral or mildly positive partial charges, except for the protonated amine group, which has high partial charges. Conversely, the lactose functionalization, bearing several –OH groups, shows a high number of charge dipoles which greatly contribute to its hydrophilicity.

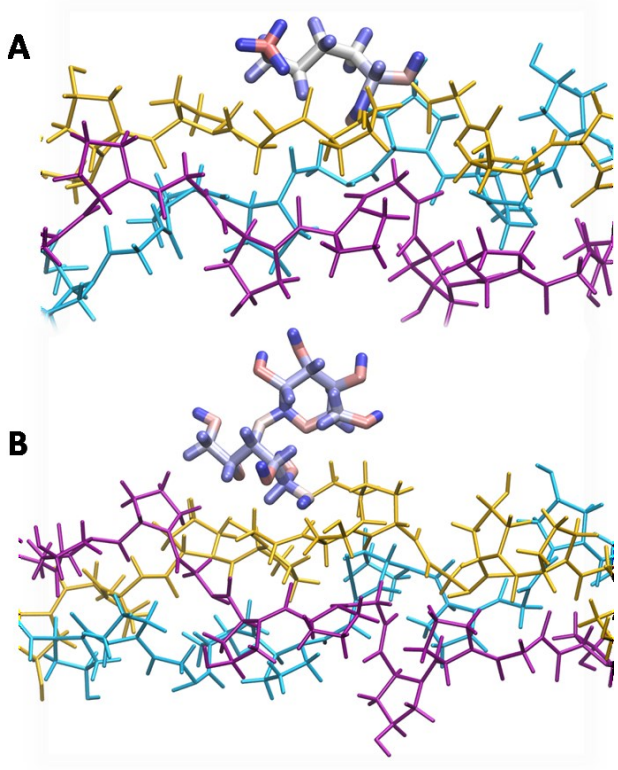

Figure 1. Configuration of lysine in untreated collagen (Panel A) and of lactose-functionalized collagen (Panel B), showing partial charge distribution (blue= positive, red= negative, white=neutral).

Chemical Neoglycosydation of collagen

Chemical functionalization of Lysine residues has been performed obtaining a "neo-glycosylated collagen" to confirm molecular dynamics simulations results. Collagen Type I from equine tendon was used for the preparation of patches by solvent casting method.[13] Neoglycosylation of collagen (Figure 2) was achieved reacting lysine side-chain amino groups with 0.06 M lactose aq. solution in 0.03 M $NaCNBH_3$ in citrate buffer (pH 6.00).

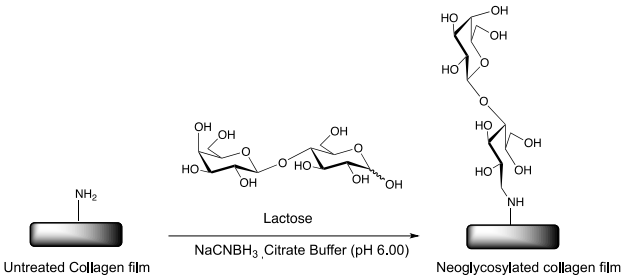

Figure 2. Scheme of the neoglycosilation procedure used to bioconjugate galactose epitope to primary amine moiety of collagen Lysine.

Morphological Characterization

The untreated collagen film observed by Tapping-Mode Atomic Force Microscopy reveals a moderately rough, grainy surface interspersed by long, straight collagen fibrils of various size and randomly oriented (see supplementary material). At higher magnification (Figure 3) a normal, regular 67 nm banding pattern is clearly visible. It must be noted that the solvent-casting method is not really suited to the formation of collagen fibrils, so that these appear haphazardly and it is normal that most molecules remain dispersed in the film.

The treatment of collagen film does not alter the appearance of the specimen (see supplementary material section), and even at higher magnification no swelling and no disaggregation were ever observed in the treated specimens. The phase shift imaging (Figure 4) reveals a somewhat cleaner surface with respect to the untreated film, with a clear-cut 67 nm cross banding and a superimposed faint longitudinal striping, corresponding to the layout of the collagen molecules within the fibril.

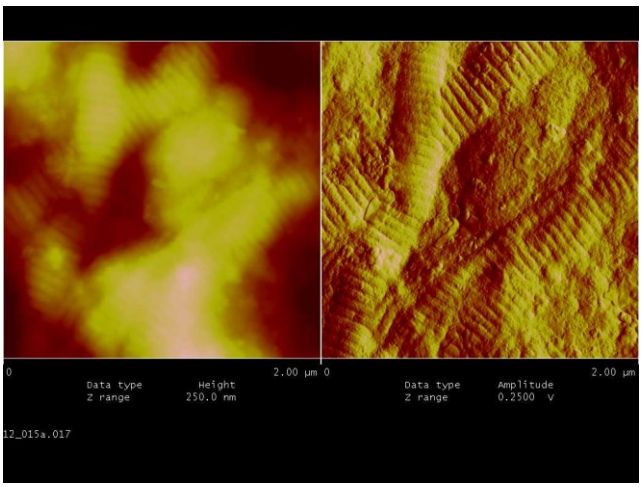

Figure 3 TMAFM micrograph of the untreated collagen film, taken in air at a scan rate of approx. 1.5 Hz. As in the following image, the left panel depicts the height-coded actual topography of the specimen while the

right panel contains a feedback signal, comparable to a derivative filter, taken from the same field of view.

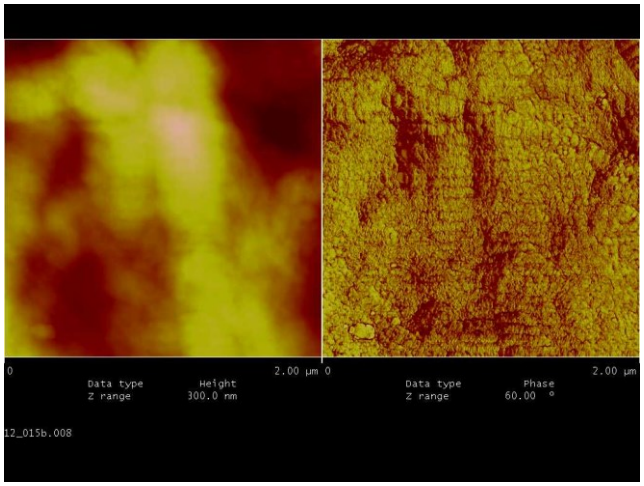

Figure 4. Visualization of neoglycosylated collagen fibrils taken simultaneously in specimen topography (left) and phase delay (right). A faint longitudinal striping appears along the fibrils in this latter panel.

Contact angle

Wettability of functionalized collagen substrates was assessed by contact angle sessile drop method. Five measurements were performed on both untreated (as control) and functionalized collagen Figure 5 shows the difference in the wettability of the two substrates, in particular glycosylated samples (Figure 5A) has a contact angle of 69,97°±0,80° that is statistically different (p <0,0001) from the value obtained for un-glycosylated samples 88,38°±0,79° (Figure 5B).

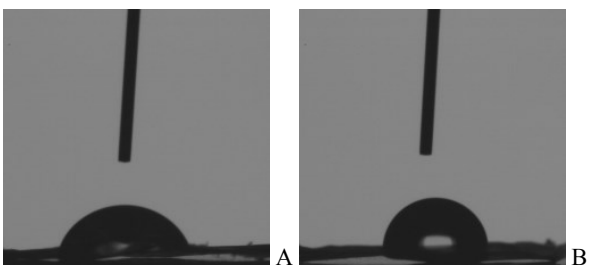

Figure 5 Contact Angle Anlysis. Glycosylated sample is shown in panel A, whereas un-glycosylated sample is shown in panel B. The drop shape analysis system was at 25°C and a drop of bidistilled water (5µl) was placed on both sample substrates.

It is possible to observe that in the case of glycosylated surfaces the wettability is significantly increased with respect to the control and that this change is statistically relevant. Moreover this result confirms the nanoscale behaviour foreseen in the preliminary molecular design phase.

Biological Function analysis

Finally, in order to assess if the linked saccharide may exploit its biological signaling functions upon recognition by its complementary receptor, preliminary Enzyme Linked Lectin Assay (ELLA)[14] on the neo-glycosylated collagen sample was performed (Figure 6). Lectins are very specific carbohydrate-recognising proteins that are commercially available conjugated to horse radish peroxidase (HRP). Collagen films, after appropriate blocking to minimize non-specific binding, were incubated with peanut lectin from *Arachys hypogaea* (PNA) specific for β-galactose labelled with HRP; after incubation, the samples were washed to remove excess of lectin. Finally, PNA-HRP-treated films were throughly washed with ultrapure water and reacted with soluble peroxidase substrate (*o*-phenylenediamine, OPD). The absorbance of the resulting surnatant was measured at 450 nm (Figure 6) indicating the presence of lectin bound to collagen surface.

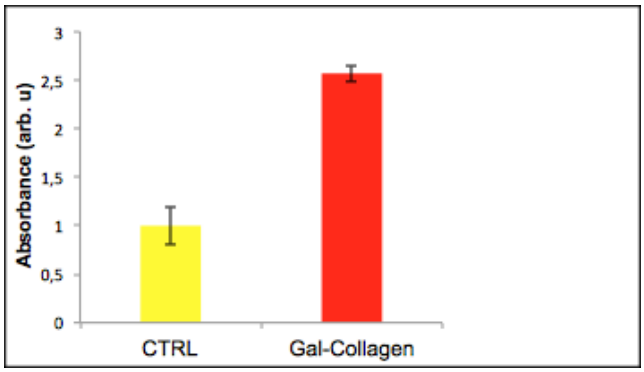

Figure 6. ELLA data for collagen functionalized with beta-galactose moieties (Gal-Collagen) recognized by peanut agglutinin. (Yellow). Negative control with by Concanvalin-A able to recognize alpha-glucose epitope.

The unfunctionalised collagen films were used in order to provide a comparison to the neoglycosylated surfaces.

Conclusions

In conclusion, collagen 2D-scaffolds have been successfully glycosylated by reductive amination on lysine side chains. Both theoretical calculation and AFM analysis highlight that the chemical glycosylation is not affecting scaffold features. ELLA biochemical assays, in addition, evidence that the galactose untit of lactose is efficiently exposed on the scaffold surface for recognition by its receptor. Since galactose is one of the saccharidic residues most commonly found on collagen glycosylation patterns, here we demonstrate that reductive amination is a valuable method to functionalize and correctly expose galactose moieties as signalling biomolecule without any detrimental effect on the scaffold structure.

Acknowledgements

This work has been supported **by Fondazione Cariplo, grant n° 2011-0270**.

Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

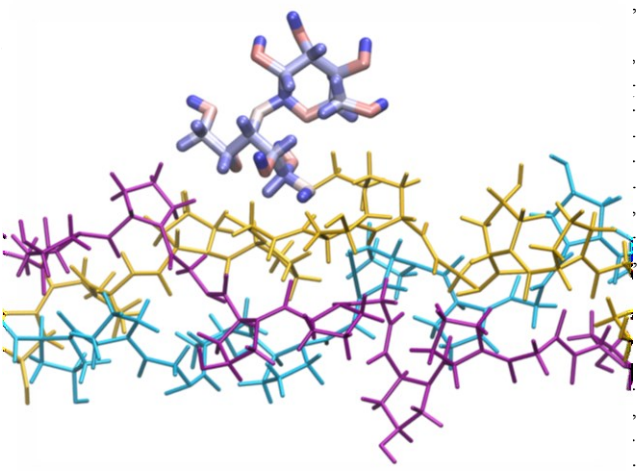
1 See for example: (a) R. S. Erdmann, H. Wennemers *Org. Biomol. Chem.*, 2012, **10**, 1982; (b) L. E. R. O'Leary, J. A. Fallas, E. L. Bakota, M. K. Kang, J. D. Hartgerink, *Nature Chem*., 2011, **3**, 821; (c) G. B. Fields *Org. Biomol. Chem*., 2010, **8**, 1237; (d) R. N. Shah, N. A. L. Shah, M. M Del Rosario, C. Hsieh, G. Nuber, S. I Stupp, *Proc. Natl. Acad. Sci. U. S. A*., 2010, **107**, 3293; (e) K. M. Hennessy *Biomaterials,* 2009, **30**, 1898.

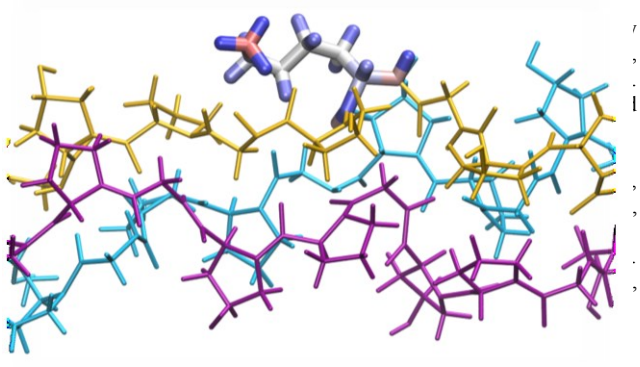
2 M. D. Shoulders, R. T. Raines, *Annu. Rev. Biochem*., 2009; *78*, 929.

3 (a) E. A. Abou Neel, L. Bozec, J. C. Knowles, O. Syed, V. Mudera, R. Day, J. Keun Hyun, *Adv. Drug Deliv. Rev.* 2013, **65**, 429; (b) A. M. Ferreira, P. Gentile, V. Chiono, G. Ciardelli, *Acta Biomater*., 2012, **8**, 3191.

4 (a) K. W. Moremen, M. Tiemeyer, A. V. Nairn, *Nature Rev* 2012, **13**, 448; (b) D. A. Tirrell, *Nature* 2004, **430**, 837; (c) Y Van Kooyk, G. A Rabinovich, *Nature Immunol*. 2008, **9**, 593; (d) K. Ohtsubo, J. D. Marth, *Cell,* 2006, **126**, 855; (e) R. Kleene, M. Schachner *Nature Rev. Neurosci*., 2004, **5**, 195.

5 See for example: (a) L. Cipolla, L. Russo, F. Taraballi, C. Lupo, D. Bini, L. Gabrielli, A. Capitoli, F. Nicotra, in Specialist Periodical Reports-Carbohydrate Chemistry, Vol. 38 (Eds: A. P. Rauter, T. Lindhorst), Royal Society of Chemistry, London, UK 2012, Ch. 17; (b) K. Chawla, T. Yu, L. Stutts, M. Yen, Z. Guan, *Biomaterials,* 2012, **33**, 6052; (c) K. E. Sapsford, W. R. Algar, L. Berti, K. B. Gemmill, B. J. Casey, E. Oh, M. H. Stewart, I. L. Medintz, *Chem. Rev*., 2013, **113**, 1904; (d) J. T. Koepsel, W. L. Murphy, *ChemBioChem* 2012, **13**, 1717; (e) A. M. Slaney, V. A. Wright, P. J. Meloncelli, K. D. Harris, L. J. West, T. L. Lowary, J. M. Buriak,





August 27th, 2013

Submission of the manuscript "*Collagen neoglycosylation at lysine residues: design, synthesis and characterization*" by Laura Russo, Alfonso Gautieri, Francesca Taraballi, Mario Raspanti, Francesco Nicotra, Laura Cipolla and Simone Vesentini

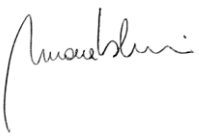
Dear Editor,

please find attached the abstract of our manuscript entitled manuscript "*Collagen neoglycosylation at lysine residues: design, synthesis and characterization*" and our motivations for your consideration for its submission as "Edge Article" to *Chemical Science.*

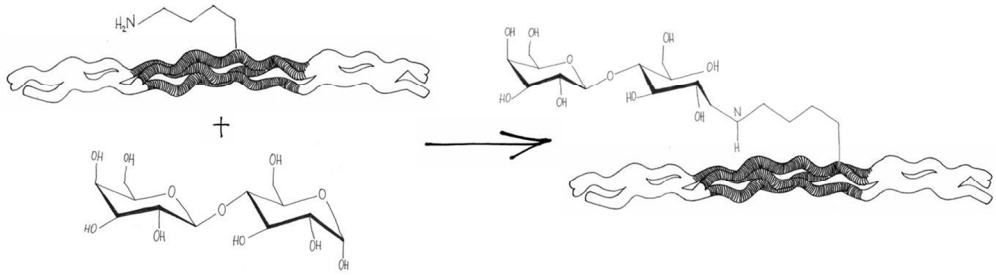
Abstract: Collagen scaffolds have been glycosylated with lactose by reductive amination at lysine side chains. AFM analysis highlights that the chemical glycosylation does not affect molecular assembly into fibrils, and increases surface wettability compared to the untreated sample, as expected by theoretical calculation. Moreover, ELLA biochemical assays show that galactose unit of lactose is efficiently exposed on the scaffold surface for recognition by its receptor and, more intriguing from a chemical perspective, for inclusion of different functional saccharidic epitopes.

Motivation: Tanks to its structural and functional characteristics collagen molecule has attracted researchers in different fields. Starting from molecular biologists whose interests regarded, as an example, the role of its repetitive structure in cell recognition, stability, or genetic pathologies; passing through biomaterialists interested in its biocompatibility and its auto-assembly feature in the building of biomimetic scaffolds for tissue engineering. More recently it has fascinated researchers working on supra-molecular structures and polymer for the possibility to conjugate collagen with artificial polymer chains, hence obtaining desired supramolecular features and behaviors. In this regard, collagen conjugation with sugar (glycation) is a less explored area although in molecular biology it is well established that collagen glycation is one the main post-transductional modification that takes place in collagen maturation. We propose a new glycation method to specifically functionalize the lysines along the collagen molecule with a lactose disaccharide. We verified the consistency of our functionalization procedure in particular assessing how modified collagen does not lose its ability to assembly in highly-ordered structure: the fibril. In particular, to assess the molecular detail of this functionalization molecular modeling analysis of a collagen-like peptide pre- and post-modification have been carried out. In our opinion, reading the text it is possible to note how this bottom up approach allowed also to drive some molecular explanations for the observed bulk properties (in particular wettability). This functionalization procedure represents an effective and innovative mean to further modify collagen molecules such as adding new functional arms and reaching new biological functions. In particular, this bio-orthogonalization strategy seems feasible and a new tool for the modification of collagen molecules to be applied with a wide range of functional modifications. In conclusion, we would like to underline that this is the first step of a concerted action that brings together bioengineers, biochemists, and molecular morphologists in the building of a more complex macromolecular collagen-based structure all made by building blocks of biological source. All the authors were involved in writing the manuscript for the part regarding their competences. We confirm that the manuscript, or its contents in some other form, has not been published previously by any of the authors and/or is not under consideration for publication in another journal at the time of submission. All authors have seen and approved the submission of the manuscript. We thank you for your kind consideration, and we are looking forward to hearing from you.

With kind regards,



Simone Vesentini



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