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DISCUSSIONS

Glycan interactions on glycocalyx mimetic surfaces: general discussion

Helena S. Azevedo, [ID] Adam B. Braunschweig, Ryan C. Chiechi, Yuri Diaz Fernandez, Jeffrey C. Gildersleeve, Kamil Godula, Laura Hartmann, Yoshiko Miura, [ID] Stephan Schmidt, W. Bruce Turnbull [ID] and Dejian Zhou

Adam Braunschweig opened discussion of the paper by Yoshiko Miura: Carbohydrate–carbohydrate interactions are poorly understood, even considered by some to be a myth because they are so weak. I was wondering if your materials could exploit or examine carbohydrate–carbohydrate interactions by using them for separations of carbohydrates, which still remain a major challenge.

Yoshiko Miura answered: The multivalent compounds are also applied to the carbohydrate–carbohydrate interaction. First of all, the carbohydrate–carbohydrate interactions are much weaker than sugar–protein interactions. It is still difficult to obtain quantitative binding constants of carbohydrate–carbohydrate interactions. The carbohydrate–carbohydrate interactions are amplified by multivalent glycopolymer compounds and carbohydrate–protein interactions.[1] In my opinion, from the quantitative measurement results, the carbohydrate–carbohydrate interactions are too weak to apply it as a functional material. (The binding constants of the glycopolymer and carbohydrate are in the order of 10^4 M^{-1}, which are too weak.) However, the continuous flow system is a useful method to estimate the carbohydrate–carbohydrate interaction.[2] Investigation of the glycopolymer monolith (porous materials) is still its infancy and should be expanded to new areas including carbohydrate–carbohydrate interactions.

1 K. Matsuura, H. Kitakouji, N. Sawada, H. Ishida, M. Kiso, K. Kitajima and K. Kobayashi, *J. Am. Chem. Soc.*, 2000, **122**, 7406–7407.
2 G. N. Misevic and M. M. Burger, *J. Biol. Chem.*, 1993, **268**, 4922–4929.

Laura Hartmann opened discussion of the paper by Kamil Godula: Did you look at different viruses attaching to the glycocalyx in your model?

Kamil Godula replied: We have not tested any viruses other than influenza A. However, we do see similar behaviour (*i.e.* enhanced clustering in the presence of a bulky glycocalyx) even for multimeric lectins, such as SNA. This suggests broader generality for the biophysical model we propose, which may extend to other viruses.

Laura Hartmann continued: Do you think, with the proposed two-step or gradient-like attachment of lectins or pathogens to the cell's glycocalyx, this can also have an impact on the specificity of these recognition events?

Kamil Godula responded: That is an interesting question and an intriguing possibility. One can imagine that two proteins with similar glycosylation may be targeted differently by a lectin or a pathogen, depending on their localization within the glycocalyx. Evidence suggests, for instance, that the influenza A virus may bind to the peripheral regions of the glycocalyx in an unproductive way and needs to translocate to glycan receptors in a different region of the glycocalyx in order to initiate internalization and infection.

Dejian Zhou opened discussion of the paper by Helena Azevedo: How does the molecular weight of hyaluronic acid affect its binding to the Pep-1 coated surface? Are there correlations between the molecular weight and amount bound?

Helena Azevedo replied: In another study,[1] we have investigated the deposition of hyaluronan with different molecular weights on the Pep-1 SAMs by QCMD, and we observed increasing mass deposition of HA with higher molecular weight.

1 X. Pang, C. O'Malley, J. Borges, M. M. Rahman, D. W. P. Collis, J. F. Mano, I. C. Mackenzie and H. S. Azevedo, Supramolecular Presentation of Hyaluronan onto Model Surfaces for Studying the Behavior of Cancer Stem Cells, *Adv. Biosyst.*, 2019, DOI: 10.1002/adbi.201900017.

Stephan Schmidt commented: Nature employs high molecular weight hyaluronic acid in the extracellular matrix to drive biological functions. How is this possible when cell assays typically show reduced interactions with high molecular weight hyaluronic acid in comparison to low molecular weight hyaluronic acid?

Helena Azevedo replied: That is a very good question. The conditions used in the *in vitro* studies do not fully replicate the *in vivo* scenario. In particular, the use of serum in cell cultures may play a role in how serum proteins interact with hyaluronic acid (HA) of different molecular weights. It may be worth investigating this in more detail.

Bruce Turnbull enquired: How do you interpret the QCMD data in terms of the arrangement of hyaluronan polysaccharide chains at the surface? Are they mostly lying parallel to the surface or extending away from the surface, and do you see different populations of weakly and strongly-bound chains?

Helena Azevedo responded: In another study[1] and using QCMD, we have determined the thickness of the HA layers using the Voigt model. We obtained values of 5–11 nm, indicating that the polymer is mostly lying parallel to the surface and not extending away from the surface. The thickness of a fully extended HA molecule of 1.5 MDa will give a thickness of $\approx$ 3.75 μm.

1 X. Pang, C. O'Malley, J. Borges, M. M. Rahman, D. W. P. Collis, J. F. Mano, I. C. Mackenzie and H. S. Azevedo, Supramolecular Presentation of Hyaluronan onto Model Surfaces for Studying the Behavior of Cancer Stem Cells, *Adv. Biosyst.*, 2019, DOI: 10.1002/adbi.201900017.

In the QCMD experiments, a washing step with saline solution (150 mM NaCl) is always done after the HA deposition. No significant changes in frequency (mass changes) are observed, indicating strongly bound chains.

Bruce Turnbull followed this by asking: What is the individual affinity of your synthetic peptides for hyaluronan?

Helena Azevedo replied: The apparent affinity of Pep-1 to hyaluronan (HA) was reported in the original paper[1] to be in the micromolar range (Kd = 1.65 μM, not very high affinity) using HA-coated beads. We have used surface plasmon resonance (SPR) with Pep-1 immobilized on a gold chip and flowing 20 kDa HA and found similar values (also in the micromolar range, unpublished data).

1 M. E. Mummert, M. Mohamadzadeh, D. I. Mummert, N. Mizumoto and A. Takashima, *J. Exp. Med.*, 2000, **192**, 769–780.

Helena Azevedo continued with the discussion of her paper: Regarding the use of 3D arrays *vs.* 2D arrays, despite the fact our work is based on 2D arrays (gold surfaces functionalized with self-assembled peptide monolayers), we have also attempted to move to 3D, using gold nanoparticles. However, Pep-1 (the peptide used in our work to bind hyaluronan) is quite hydrophobic and promoted aggregation of the gold nanoparticles, requiring further modification of the peptide sequence near the gold surface. Similarly, we are interested in translating these 2D model surfaces to develop 3D hydrogels and recreate tumor microenvironments. Many tumors are rich in hyaluronan (HA) and Pep-1 could be used as a binding motif to capture HA in a 3D hydrogel.

Yuri Diaz Fernandez reopened discussion of the paper by Yoshiko Miura: It is impressive that you can cover a considerable range of pore sizes by just changing the alcohol used during the polymerization process. Could you please comment on the mechanism behind the effect of the alcohol?

Yoshiko Miura responded: The porous glycopolymer was prepared based on the phase separation of the polymer. There are several methods of porous polymer preparation. Our current method is polymerization-induced phase separation (PIPS), and Svec and Fréchet reported the preparation of porous polyacrylamide by PIPS in 1997.[1] The monomers have higher solubility before polymerization because the molecular weight of the monomer is small. The glycopolymer has a lower solubility in the solvent. The solvent used was DMSO and alcohols were used as porogens. Since polyacrylamide is a water soluble polymer, the glycopolymer with polyacrylamide was also water soluble. The polymers were soluble in the polar solvent DMSO, but were not soluble in the less polar alcohol solvent. It was interesting that uniform porous polymers could be prepared with this simple preparation method. Considering the mechanism, there is a possibility that the interface affects the polymer phase separation, but it was not affected much due to the polymer structure.

1 S. Xie, F. Svec and J. M. J. Fréchet, *J. Polym. Sci. A*, 1997, **35**, 1013–1021.

Laura Hartmann asked: Can you also use PIPS to create micro- or nano-particles with a porous structure?

Yoshiko Miura responded: The PIPS mechanism is utilized for the preparation of various polymeric materials, including particles. The method is applied not only at the experimental level, but also to the commercial product. For example, see N. Tsujioka, N. Ishizuka, N. Tanaka, T. Kubo and K. Hosoya, *J. Polym. Sci. A.*, 2008, **46**, 3272–3281. Both the bulk and particle forms were obtained.

Jeffrey Gildersleeve continued the discussion of Kamil Godula's paper: Are the effects of crowding on rate and affinity different when a lectin or virus binds a glycolipid *versus* a glycoprotein? For example, glycolipids are much closer to the cell membrane, while glycans on a glycoprotein might be found closer to the external edge/boundary of a cell. In addition, glycolipid mobility can be quite different compared to a glycan on a glycoprotein.

Kamil Godula answered: We have not probed this effect experimentally but my expectation is that this may be the case. We are certainly observing differences in the rate and affinity of influenza A *vs.* lectin binding as a function of glycopolymer size and density in glycan array studies.

Adam Braunschweig addressed all the presenters: As we know, for glycopolymers, binding avidity and signal transduction following binding are responses that are sensitively dependent on backbone stiffness. This is a material property of which the role is notoriously difficult to understand. Could your synthetic systems be used to study how backbone stiffness affects avidity? Also, in what ways may your model polymers not accurately reflect how binding occurs biologically?

Yoshiko Miura responded: This is an important point in the design of glycopolymers. The physical properties of the ligands are very important for molecular recognition, including in glycopolymer. The design of glycopolymers has been varied, and facile preparation is preferred. We have reported the effect of the physical properties of a glycopolymer nanogel.[1] As Professor Braunschweig suggested, the stiffness of the polymer affects the binding of the glycopolymer and its molecular recognition.

In the glycopolymer, molecular recognition is controlled by enthalpy and entropy, where the enthalpy gain is mainly from the sugar–protein interaction (*i.e.* the hydrogen bonding of sugar–protein). The polymer stiffness affects the molecular recognition due to an entropic effect. Although the comparison between the natural system and the artificial ligand (glycopolymer) is important, only using a limited method can differences be verified. For example, the measurement of the specificity and the binding affinity are representative examples. It is difficult to know the accuracy of the artificial glycopolymer system. However, if various glycopolymers with different multivalency and physical properties are synthesized and examined, the findings obtained from these materials should be considered as models to represent phenomena occurring in nature.

1 Y. Hoshino, M. Nakamoto and Y. Miura, *J. Am. Chem. Soc.*, 2012, **134**, 15209–15212.

Helena Azevedo added: We have synthesized hyaluronan (HA) glycopolymers whereby HA sugars were grafted on a synthetic rigid polymer backbone (manuscript in preparation). We have performed small angle X-ray scattering (SAXS) on solutions of the glycopolymers and their scattering patterns differ from those of native HA, which exhibits a pattern typical of salt-free poly-electrolyte solutions. We have also studied the binding of the HA glycopolymers to CD44, the major receptor for HA, using surface plasma resonance (SPR) and observed binding to CD44. I believe glycopolymers can be used as probes to dissect the role of backbone stiffness and monosaccharide composition/presentation in protein–carbohydrate interactions of natural systems.

Ryan Chiechi returned to the discussion of Kamil Godula's paper: How is the post-polymerization chemistry of the poly(epichlorohydrin)s characterized? The reaction schemes state that the yields are quantitative, however, there will be some fraction of missing pendant groups. Due to the nature of the polymers and that their effects are studied at the level of single polymer chains, might these vacancies be important?

Kamil Godula replied: The extent of glycosylation is determined by ^{1}H-NMR and by IR analysis for the conversion of pendant azide chains. We observed complete disappearance of the characteristic azide stretch in the IR spectra of glycopolymers, which we interpreted as complete conversion, seeing as we can detect the presence of even a single end-chain azide group in the precursor polymer backbone. The level of glycosylation will influence the physical properties of the polymers (*i.e.* persistence length, stiffness, *etc.*)

Ryan Chiechi said: Polymer chains decorated with sugars were described as not rod like and yet not globular. Have their dynamics been modeled in detail?

Kamil Godula answered: Some studies on the effects of glycosylation on biopolymer stiffness and persistence length have been carried out in the past, particularly in the context of mucin glycoproteins and, recently, also in synthetic NCA glycopolypeptide polymers. The ability to model and predict these properties in synthetic polymers would be great to have in order to guide glycopolymer design.

Ryan Chiechi commented: When discussing the self-assembly of, presumably, very sterically crowded polymers at the surfaces of cells and lipid bilayers, the mechanistic explanations for their effects were very mechanical and invoked cartoons of macromolecules literally getting in each other's way (DOI: 10.1039/c9fd00024k). It would therefore seem that bulk descriptors, such as Young's modulus, do not capture the salient mechanical properties of the individual polymer chains. What is the best way to describe these properties?

Kamil Godula answered: I think a recent paper by Paszek, extending the physical principles applied to surface-bound polymer brushes, provides a nice quantitative framework for the mucinous glycocalyx.[1]

1 C. R. Shurer, J. C.-H. Kuo, L. M. Roberts, J. G. Gandhi, M. J. Colville, T. A. Enoki, H. Pan, J. Su, J. M. Noble, M. J. Hollander, J. P. O'Donnell, R. Yin, K. Pedram, L. Möckl, L. F. Kourkoutis, W. E. Moerner, C. R. Bertozzi, G. W. Feigenson, H. L. Reesink and M. J. Paszek, *Cell*, 2019, **177**, 1757–1770.

Jeffrey Gildersleeve remarked: You describe some interesting effects of crowding on the rates of binding and affinities. Have you evaluated the effects of proteins with significant differences in size, such as IgG *versus* IgM antibodies? Do you think crowding will influence the recognition of cells by different isotypes of antibodies?

Kamil Godula replied: We have not compared proteins of different sizes but we did compare lectins to whole influenza A viruses. The effects on virus binding are more pronounced in the presence of an extended glycocalyx (long polymers), while lectins are influenced more by the glycocalyx density (short polymers).

Conflicts of interest

There are no conflicts to declare.