Sialic acid-dependent self-agglutination is a barrier to influenza virosome assembly

Ashraf S Yousif, Maya Sangesland, Daniel Lingwood
The Ragon Institute of MGH, MIT and Harvard;

Correspondence
dlingwood@mgh.harvard.edu

Disciplines
Biophysics
Biochemistry
Virology

Keywords
Membrane
Multivalency

Type of Observation
Standalone

Type of Link
Orphan Data

Submitted Jan 16, 2016
Published Feb 19, 2016

Triple Blind Peer Review
The handling editor, the reviewers, and the authors are all blinded during the review process.

Full Open Access
Supported by the Velux Foundation, the University of Zurich, and the EPFL School of Life Sciences.

Abstract
Enhancing the affinity of weak interactions through multivalency and avidity is a cornerstone of many biological recognition processes. Through assembly of influenza hemagglutinin (HA) virosomes, we found that high densities of this spike protein, normally needed to bind the primary viral receptor sialyl oligosaccharide (SO), also promote self-agglutination following two-dimensional membrane presentation of HA. Sterically blocking HA-SO binding prevented this and allowed for virosome formation, predicting that the sialidases normally present in the influenza virion are important for displaying HA at high density.

Introduction
Biological systems rely on sets of evolutionarily selected weak interactions that maintain the functional organization of macromolecules [3] [4] [5]. This property ensures the reversibility of interactions and the emergence of collective behaviors [6] [7] [8]. One key solution that enhances affinity within this energetic landscape is multivalent interaction [9] [10] [11]. In this case, the accumulated strength of multiple affinities will generate a functional affinity far stronger than that provided by the monomer [12] [13] [14]. This property can be critical for receptor-ligand interactions, as illustrated by the first step of infection with influenza virus, where the virus attaches to the surface of a bronchial epithelial cell. The primary receptor for this interaction is cell surface sialyl-oligosaccharide (SO) which is recognized by a receptor-binding site (RBS) on the influenza spike protein hemagglutinin (HA) with an extraordinarily weak affinity (millimolar-range equilibrium dissociation constant, kD) [15]. Alone this would offer little binding specificity; however, functional affinity for a target cell is achieved by multivalent display, wherein densely packed HA trimers occupy much of the virion surface, up to 2–4 copies per 100 nm^2 or 600–1200 copies per virus particle [9] [15] [16].

Objective
We sought to define, through influenza virosome construction, [17] [18] the physical constraints that must be addressed to accommodate the display of HA at high density.

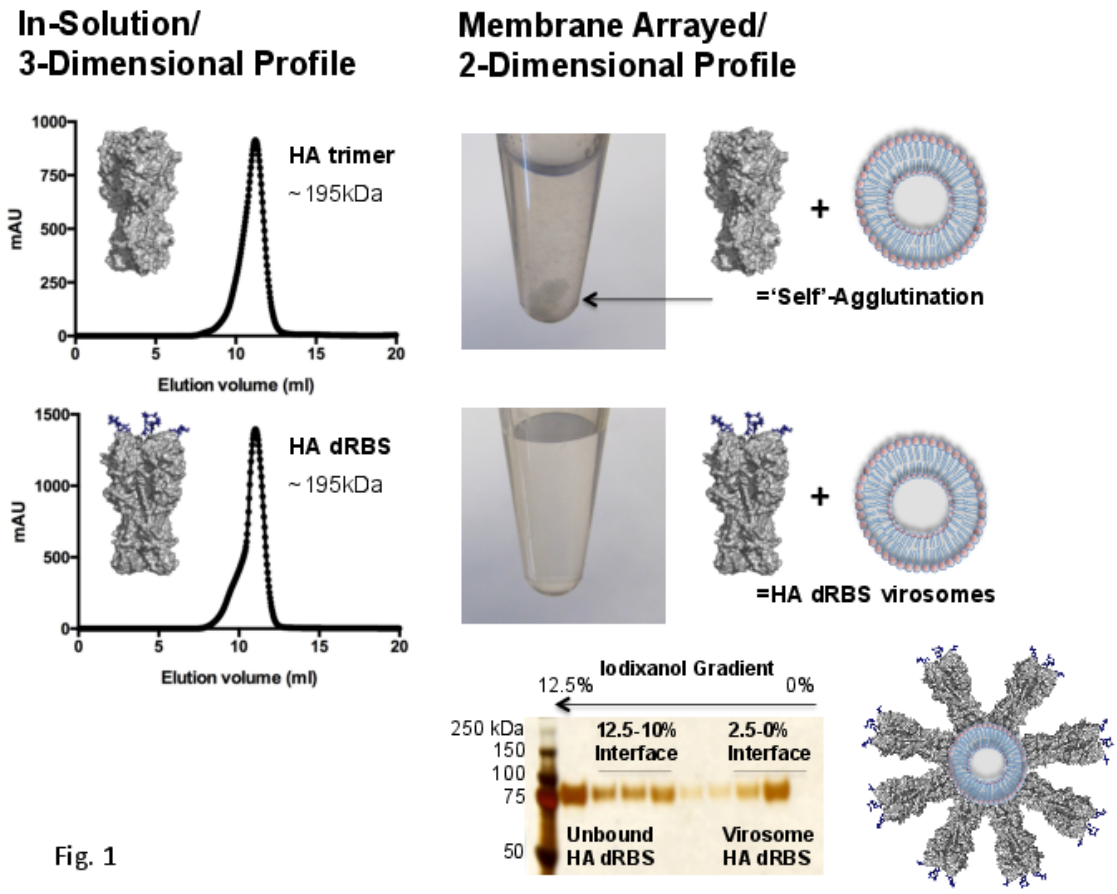

Fig. 1

a

Figure Legend

Figure 1. Dimensionality regulates the 'self'-agglutination of influenza HA. WT HA is trimeric in solution as seen by size exclusion chromatography (upper left) but induces self-agglutination when arrayed on a two-dimensional membrane surface (upper right). This agglutination prevents subsequent virosome production and isolation by equilibrium centrifugation. Insertion of a SO-blocking glycan (blue) within the HA receptor-binding site (HA dRBS) preserves trimeric structure (lower left) and allows for the generation of virosomes following membrane presentation (lower right). Virosomes are separated by flotation in an iodixanol density gradient; presented are the gradient fractions following SDS PAGE and silver staining. HA floating at the 2.5–0% interface represents proteoliposome/virosome displayed trimer [1] [2].

Plasmids encoding the extracellular domain of HA (Influenza A/New Calendonia/20/1999) C-terminally fused to the trimeric Foldon of T4 fibritin and a hexahistidine affinity tag proteins [19] were transfected into the human embryonic kidney cell line 293F and isolated from expression supernatants 96 h post-transfection. HA trimers, either wildtype (WT) or a mutant HA that masks the RBS by glycosylation at residue 190 (dRBS HA), were purified as previously described [19] [1] [2]. The trimeric assembly of these viral proteins was confirmed by size exclusion FPLC on a prepacked Superdex 200 10/300 column (Life Technologies). Virosome construction was as described [1] [2]. Briefly, 1 g 1,2-dioleoyl-sn-glycero-3-phosphocholine: 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid)] succinyl [DOPC: DGS-NTA(Ni)] (Avanti Polar Lipids Inc.) in a 1:1 molar ratio was evaporated under a stream of nitrogen for 1 h. The lipid film was then rehydrated in 1 mL liposome buffer [50 mM HEPES, 150 mM NaCl, pH 7.25 (HBS)] and shaken for 40 min, above the Tm of the lipid mixture.

The suspension was then subjected to 10 freeze-thaw cycles and then extruded 21 times through a 100 nm polycarbonate membrane using a mini-extruder (Avanti Polar Lipids Inc.). HA virosomes/proteoliposomes were produced by incubating the resultant liposomes with either His-tagged HA or His-tagged DRBS HA (HA trimer-lipid molar ratio of 1/900). To isolate HA-virosomes, the sample was adjusted to 15% iodixanol (in 1.25 mL HBS) and overlaid with 1.75, 0.5, and 0.5 mL of 10%, 2.5%, and 0% iodixanol in HBS, respectively. Samples were then centrifuged at 200,000 *g* in a TH660 rotor (Sorvall) for 2 h.

Results & Discussion

We found that recombinant WT HA trimer was free of aggregation or self-agglutination in solution (Fig. 1). However, when arrayed on the two-dimensional surface provided by preformed liposomes, we noted immediate agglutination of the liposomes, preventing HA virosome formation (Fig. 1). While this enhancement of HA lectin activity underscores the importance of dimensionality and valency in generating biological work from weakly interacting proteins, it also highlights the difficulty of arraying HA at high occupancy, an architecture that is critical for viral infectivity [9] [15] [16]. Insertion of a single glycan in the viral RBS (HA dRBS) prevented this self-agglutination and allowed for the production of HA-virosomes, with the virosome fraction distributing at the 2.5%-0% iodixanol interface following equilibrium centrifugation (Fig. 1). The dRBS mutation did not affect trimeric assembly of the protein (Fig. 1) and has been previously established to produce the same SO-binding inhibition as is seen when the WT HA trimer is incubated with soluble SO competitor [1] [19]. This indicated that the two-dimensional self-agglutination exhibited by WT HA virosomes was SO-dependent. Interestingly, influenza viruses deficient in the SO-cleaving viral enzyme neuramindase do not assemble [20] [21]. It has generally been presumed that this enzyme is required for viral release from the cell surface through cleavage of the SO substrate [22] [23]. However, our data suggests that its activity may also be important for overcoming the SO-dependent obstacle to displaying HA at high density, the key multivalent HA presentation that allows this virus to generate sufficient affinity to engage a target cell [9] [15]. This highlights an unusual dichotomy wherein the very same avidity principle used to generate biochemical specificity for a weak affinity target also impedes the generation of the multivalent solution used to achieve that specificity, likely necessitating the presence of additional viral sialidases. A high surface occupancy of HA is also important for strengthening immune responses through virosome display and multivalent subunit vaccines [18] [17]. Multivalent antigen display has long been implicated in the enhancement of vaccine immunogenicity [24] [25] and thus our findings also illustrate the importance of RBS-blocking mutations in the design of arrayed HA immunogens. Given that the RBS is well conserved across influenza viral strains [15], our RBS-blocking mutation can be readily applied to prevent self-agglutination by any number of HA subtypes when displayed in virosome or other multivalent/particulate vaccine format.

Additional Information

Methods

Plasmids encoding the extracellular domain of HA (Influenza A/New Calendonia/20/1999) C-terminally fused to the trimeric Foldon of T4 fibritin and a hexahistidine affinity tag proteins [19] were transfected into the human embryonic kidney cell line 293F and isolated from expression supernatants 96 h post-transfection. HA trimers, either wildtype (WT) or a mutant HA that masks the RBS by glycosylation at residue 190 (dRBS HA), were purified as previously described [19] [1] [2]. The trimeric assembly of these viral proteins was confirmed by size exclusion FPLC on a prepacked Superdex 200 10/300 column (Life Technologies). Virosome construction was as described [1] [2]. Briefly, 1 g 1,2-dioleoyl-sn-glycero-3-phosphocholine: 1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid)] succinyl [DOPC: DGS-NTA(Ni)] (Avanti Polar Lipids Inc.) in a 1:1 molar ratio was evaporated under a stream of nitrogen for 1 h. The lipid film was then rehydrated in 1 mL liposome buffer [50 mM HEPES, 150

mM NaCl, pH 7.25 (HBS)] and shaken for 40 min, above the Tm of the lipid mixture. The suspension was then subjected to 10 freeze-thaw cycles and then extruded 21 times through a 100 nm polycarbonate membrane using a mini-extruder (Avanti Polar Lipids Inc.). HA virosomes/proteoliposomes were produced by incubating the resultant liposomes with either His-tagged HA or His-tagged DRBS HA (HA trimer-lipid molar ratio of 1/900). To isolate HA-virosomes, the sample was adjusted to 15% iodixanol (in 1.25 mL HBS) and overlaid with 1.75, 0.5, and 0.5 mL of 10%, 2.5%, and 0% iodixanol in HBS, respectively. Samples were then centrifuged at 200,000 *g* in a TH660 rotor (Sorvall) for 2 h.

Supplementary Material

Please see https://sciencematters.io/articles/201601000013.

Funding Statement

This work was supported by the following awards to D.L.: a Harvard University CFAR grant (P30 AI060354); the William F. Milton Fund; the Gilead Sciences Research Scholars Program in HIV; and an Broad-Ragon ENDHIV Catalytic Grant.

Acknowledgements

The authors thank Patrick McTamney for assistance with generation of the HA structures.

Ethics Statement

Not applicable.

Citations

[1] Daniel Lingwood et al. "Structural and genetic basis for development of broadly neutralizing influenza antibodies". In: *Nature* 489.7417 (Aug. 2012), pp. 566–570. DOI: 10.1038/nature11371. URL: http://dx.doi.org/10.1038/nature11371.

[2] Grant C Weaver et al. "In vitro reconstitution of B cell receptor–antigen interactions to evaluate potential vaccine candidates". In: *Nature Protocols* 11.2 (Jan. 2016), pp. 193–213. DOI: 10.1038/nprot.2016.009. URL: http://dx.doi.org/10.1038/nprot.2016.009.

[3] E. H. McConkey. "Molecular evolution, intracellular organization, and the quinary structure of proteins." In: *Proceedings of the National Academy of Sciences* 79.10 (May 1982), pp. 3236–3240. DOI: 10.1073/pnas.79.10.3236. URL: http://dx.doi.org/10.1073/pnas.79.10.3236.

[4] Gierasch Lila M and Gershenson Anne. "Post-reductionist protein science, or putting Humpty Dumpty back together again". In: *Nature Chemical Biology* 5.11 (Nov. 2009), pp. 774–777. DOI: 10.1038/nchembio.241. URL: http://dx.doi.org/10.1038/nchembio.241.

[5] Anna Jean Wirth and Martin Gruebele. "Quinary protein structure and the consequences of crowding in living cells: Leaving the test-tube behind". In: *BioEssays* 35.11 (Aug. 2013), pp. 984–993. DOI: 10.1002/bies.201300080. URL: http://dx.doi.org/10.1002/bies.201300080.

[6] P. Chien and L. M. Gierasch. "Challenges and dreams: physics of weak interactions essential to life". In: *Molecular Biology of the Cell* 25.22 (Nov. 2014), pp. 3474–3477. DOI: 10.1091/mbc.e14-06-1035. URL: http://dx.doi.org/10.1091/mbc.e14-06-1035.

[7] Marco Bischof and Emilio Del Giudice. "Communication and the Emergence of Collective Behavior in Living Organisms: A Quantum Approach". In: *Molecular Biology International* 2013 (2013), pp. 1–19. DOI: 10.1155/2013/987549. URL: http://dx.doi.org/10.1155/2013/987549.

[8] L.Gwenn Volkert. "Enhancing evolvability with mutation buffering mediated through multiple weak interactions". In: *Biosystems* 69.2-3 (May 2003), pp. 127–142. DOI: 10.1016/s0303-2647(02)00136-3. URL: http://dx.doi.org/10.1016/s0303-2647(02)00136-3.

[9] Mathai Mammen, Seok-Ki Choi, and George M. Whitesides. "Polyvalent Interactions in Biological Systems: Implications for Design and Use of Multivalent Ligands and Inhibitors". In: *Angewandte Chemie International Edition* 37.20 (Nov. 1998), pp. 2754–2794. DOI: 10.1002/(sici)1521-3773(19981102)37:20<2754::aid-anie2754>3.3.co;2-v. URL: http://dx.doi.org/10.1002/(sici)1521-3773(19981102)37:20%3C2754::aid-anie2754%3E3.3.co;2-v.

[10] Laura L Kiessling, Jason E Gestwicki, and Laura E Strong. "Synthetic multivalent ligands in the exploration of cell-surface interactions". In: *Current Opinion in Chemical Biology* 4.6 (Dec. 2000), pp. 696–703. DOI: 10.1016/s1367-5931(00)00153-8. URL: http://dx.doi.org/10.1016/s1367-5931(00)00153-8.

[11] Narayanaswamy Jayaraman. "Multivalent ligand presentation as a central concept to study intricate carbohydrate–protein interactions". In: *Chemical Society Reviews* 38.12 (2009), p. 3463. DOI: 10.1039/b815961k. URL: http://dx.doi.org/10.1039/b815961k.

[12] Tarun K. Dam and C. Fred Brewer. "Effects of Clustered Epitopes in Multivalent Ligand-Receptor Interactions †". In: *Biochemistry* 47.33 (Aug. 2008), pp. 8470–8476. DOI: 10.1021/bi801208b. URL: http://dx.doi.org/10.1021/bi801208b.

[13] Valentin Wittmann. "Synthetic Approaches to Study Multivalent Carbohydrate—Lectin Interactions". In: *ChemInform* 36.31 (Aug. 2005). DOI: 10.1002/chin.200531269. URL: http://dx.doi.org/10.1002/chin.200531269.

[14] Gestwicki Jason E. et al. "Influencing Receptor–Ligand Binding Mechanisms with Multivalent Ligand Architecture". In: *Journal of the American Chemical Society* 124.50 (Dec. 2002), pp. 14922–14933. DOI: 10.1021/ja027184x. URL: http://dx.doi.org/10.1021/ja027184x.

[15] John J. Skehel and Don C. Wiley. "Receptor Binding and Membrane Fusion in Virus Entry: The Influenza Hemagglutinin". In: *Annual Review of Biochemistry* 69.1 (June 2000), pp. 531–569. DOI: 10.1146/annurev.biochem.69.1.531. URL: http://dx.doi.org/10.1146/annurev.biochem.69.1.531.

[16] Ming Luo. "Influenza Virus Entry". In: *Viral Molecular Machines* (Nov. 2011), pp. 201–221. DOI: 10.1007/978-1-4614-0980-9_9. URL: http://dx.doi.org/10.1007/978-1-4614-0980-9_9.

[17] Jan Wilschut. "Influenza vaccines: The virosome concept". In: *Immunology Letters* 122.2 (Feb. 2009), pp. 118–121. DOI: 10.1016/j.imlet.2008.11.006. URL: http://dx.doi.org/10.1016/j.imlet.2008.11.006.

[18] Anke Huckriede et al. "The virosome concept for influenza vaccines". In: *Vaccine* 23 (July 2005), S26–S38. DOI: 10.1016/j.vaccine.2005.04.026. URL: http://dx.doi.org/10.1016/j.vaccine.2005.04.026.

[19] J. R. R. Whittle et al. "Flow Cytometry Reveals that H5N1 Vaccination Elicits Cross-Reactive Stem-Directed Antibodies from Multiple Ig Heavy-Chain Lineages". In: *Journal of Virology* 88.8 (Feb. 2014), pp. 4047–4057. DOI: 10.1128/jvi.03422-13. URL: http://dx.doi.org/10.1128/jvi.03422-13.

[20] B. J. Chen et al. "Influenza Virus Hemagglutinin and Neuraminidase, but Not the Matrix Protein, Are Required for Assembly and Budding of Plasmid-Derived Virus-Like Particles". In: *Journal of Virology* 81.13 (May 2007), pp. 7111–7123. DOI: 10.1128/jvi.00361-07. URL: http://dx.doi.org/10.1128/jvi.00361-07.

[21] Jeremy S. Rossman and Robert A. Lamb. "Influenza virus assembly and budding". In: *Virology* 411.2 (Mar. 2011), pp. 229–236. DOI: 10.1016/j.virol.2010.12.003. URL: http://dx.doi.org/10.1016/j.virol.2010.12.003.

[22] Debi P. Nayak, Eric Ka-Wai Hui, and Subrata Barman. "Assembly and budding of influenza virus". In: *Virus Research* 106.2 (Dec. 2004), pp. 147–165. DOI: 10.1016/j.virusres.2004.08.012. URL: http://dx.doi.org/10.1016/j.virusres.2004.08.012.

[23] Debi P. Nayak et al. "Influenza virus morphogenesis and budding". In: *Virus Research* 143.2 (Aug. 2009), pp. 147–161. DOI: 10.1016/j.virusres.2009.05.010. URL: http://dx.doi.org/10.1016/j.virusres.2009.05.010.

[24] H M Dintzis, R Z Dintzis, and B Vogelstein. "Molecular determinants of immunogenicity: the immunon model of immune response". In: *Proceedings of the National Academy of Sciences* 73.10 (Oct. 1976), pp. 3671–3675. DOI: 10.1073/pnas.73.10.3671. URL: http://dx.doi.org/10.1073/pnas.73.10.3671.

[25] B. MOREIN et al. "Effective subunit vaccines against an enveloped animal virus". In: *Nature* 276.5689 (Dec. 1978), pp. 715–718. DOI: 10.1038/276715a0. URL: http://dx.doi.org/10.1038/276715a0.