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

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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 nm² or 600–1200 copies per virus particle [9] [15] [16].

Objective
We sought to define, through influenza virome construction, [17] [18] the physical constraints that must be addressed to accommodate the display of HA at high density.
**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-dioleyl-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 neuraminidase 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
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Supplementary Material
Please see https://sciencematters.io/articles/201601000013.

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Ethics Statement
Not applicable.

Citations


