

# Thermoplasmonic Vesicle Fusion Reveals Membrane Phase Segregation of Influenza Spike Proteins

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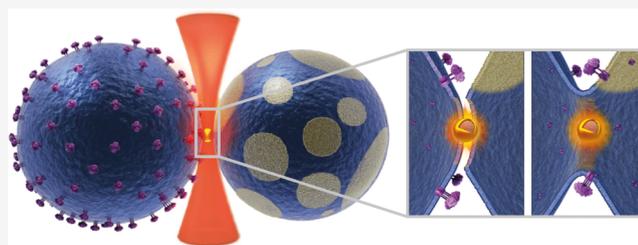
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**ABSTRACT:** Many cellular processes involve the lateral organization of integral and peripheral membrane proteins into nanoscale domains. Despite the biological significance, the mechanisms that facilitate membrane protein clustering into nanoscale lipid domains remain enigmatic. In cells, the analysis of membrane protein phase affinity is complicated by the size and temporal nature of ordered and disordered lipid domains. To overcome these limitations, we developed a method for delivering membrane proteins from transfected cells into phase-separated model membranes that combines optical trapping with thermoplasmonic-mediated membrane fusion and confocal imaging. Using this approach, we observed clear phase partitioning into the liquid disordered phase following the transfer of GFP-tagged influenza hemagglutinin and neuraminidase from transfected cell membranes to giant unilamellar vesicles. The generic platform presented here allows investigation of the phase affinity of any plasma membrane protein which can be labeled or tagged with a fluorescent marker.

**KEYWORDS:** *thermoplasmonic membrane fusion, lipid raft, viral spike proteins, neuraminidase, hemagglutinin, influenza A virus*



Lipid domains play important roles in the organization of the cellular plasma membrane and hence control a number of processes<sup>1,2</sup> ranging from membrane trafficking<sup>3</sup> to apoptosis.<sup>4,5</sup> Additionally, they are implicated in a number of diseases including cancer emergence and invasion<sup>6,7</sup> and cardiovascular diseases.<sup>8</sup> Moreover, the discovery of a high level of cholesterol and saturated lipids in the envelope of HIV<sup>9</sup> together with the finding that ordered membrane domains play a role in pathogenic microorganisms<sup>10</sup> supports the hypothesis of a modulatory role of lipid domains in host–pathogen interactions<sup>11</sup> including virus entry<sup>12</sup> and budding.<sup>13</sup>

The proliferation of many enveloped viruses is intimately dependent on the structure and organization of the viral proteins in the plasma membrane. For influenza viruses, clustering of hemagglutinin (HA) and neuraminidase (NA) in the plasma membrane is crucial for the assembly and budding of progeny virions. The mechanism behind the lateral organization of proteins in the plasma membrane remains enigmatic, but the plasma membrane lipids have been proposed to be responsible for recruitment of transmembrane proteins to nanoscale budding sites of virus infected cells.<sup>14,15</sup> The idea of lipids being responsible for clustering virus proteins at the cell surface is appealing, and indeed colocalization, and resulting crowding, of viral envelope proteins with massive ectodomain heads could produce an asymmetric lateral pressure across the membrane capable of driving bending and thus contribute to viral budding.<sup>16,17</sup>

Eukaryotic cell membranes have been proposed to form dynamic lipid raft structures enriched with proteins, allowing the cells to perform lateral organization of proteins into nanoscale domains.<sup>18</sup> The presence of rafts, initially verified using disputed detergent resistant methods, has been suggested as the driving mechanism for recruitment of viral proteins to the budding site.<sup>2</sup> Indeed, it was recently shown that oligomerization of GAG proteins involved in HIV virus facilitates formation of ordered lipid domains which recruited viral proteins to the budding site.<sup>19</sup> Such organization is critical for cell functions considering that membrane proteins constitute up to one-third of a mammalian cell proteome<sup>20,21</sup> and because the plasma membrane contains roughly 30000 proteins per  $\mu\text{m}^2$ , populating 30–55% of the membrane area.<sup>22</sup> Enriched in cholesterol and sphingolipids, lipid-raft domains could provide sorting platforms for both transmembrane and peripheral proteins possibly including viral proteins.<sup>4,23–29</sup>

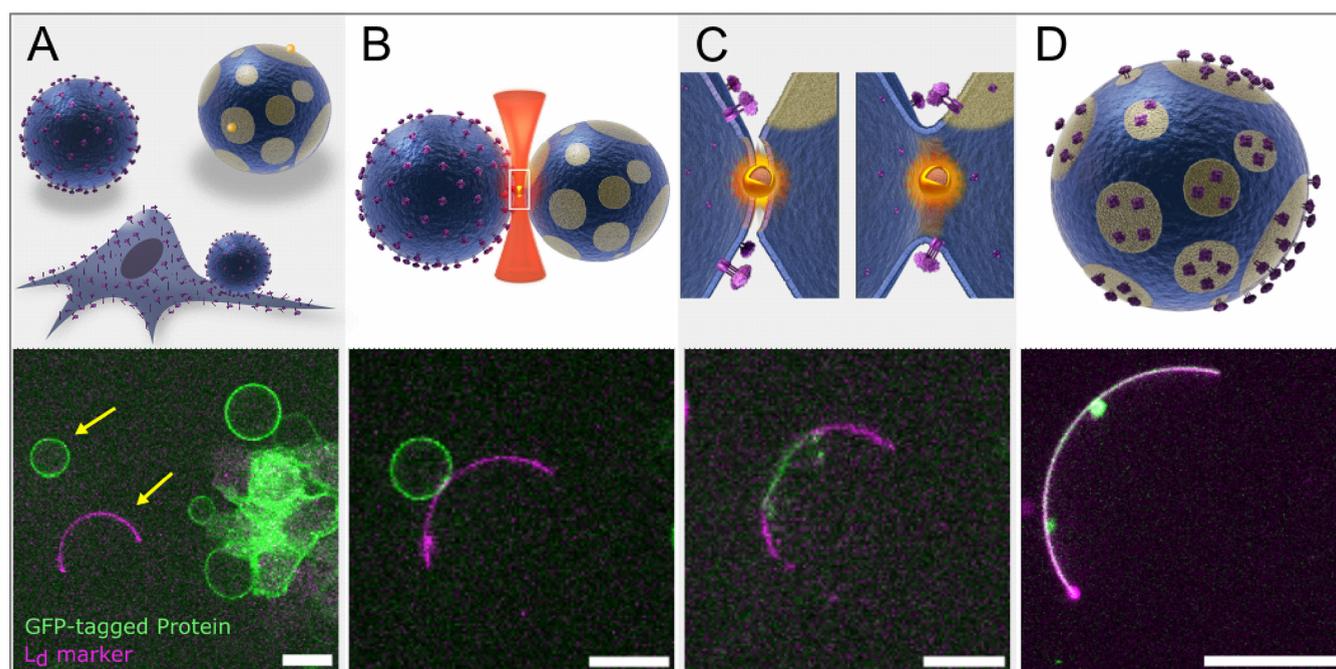
The highly dynamic structure (milliseconds) and very small size (10–200 nm) of membrane domains make visual detection and investigation of rafts difficult.<sup>30</sup> The current

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**Figure 1.** In vitro thermoplasmonic-based delivery of integral membrane proteins to phase-segregated model membranes. (A) HEK293T cells are transfected to express the protein of interest, here GFP-tagged hemagglutinin transmembrane domain (HA-TMD) with the ectodomain labeled with a GFP. In addition, they are treated with reagent that results in the production of GPMVs containing the expressed transmembrane protein in its correct orientation. Phase-separated giant unilamellar vesicles (GUVs) are also added to the cell culture. As the fusion mediator, 150 nm streptavidin conjugated gold nanoshells (AuNSs) are linked to the GUVs through the biotin–streptavidin interaction. Prior to fusion, a GPMV is grabbed by optical tweezers and brought in to close proximity of a GUV (yellow arrows). (B) Irradiation of the GPMV–GUV interface by the NIR laser attracts a AuNS into the laser focus where it produces local and transient heat sufficient to fuse the apposing membranes. (C) The process of lipid mixing following fusion. (D) The resultant vesicle retains its ordered and disordered phases, and the HA-TMD partitions into the liquid disordered phase (marked by 18:1 Liss Rhod. PE). The different colors in the schematics in (A–D, top panels) represent phases with different lipid order. The images have been adjusted in brightness and contrast for better visualization. Scale bars are 15  $\mu\text{m}$ .

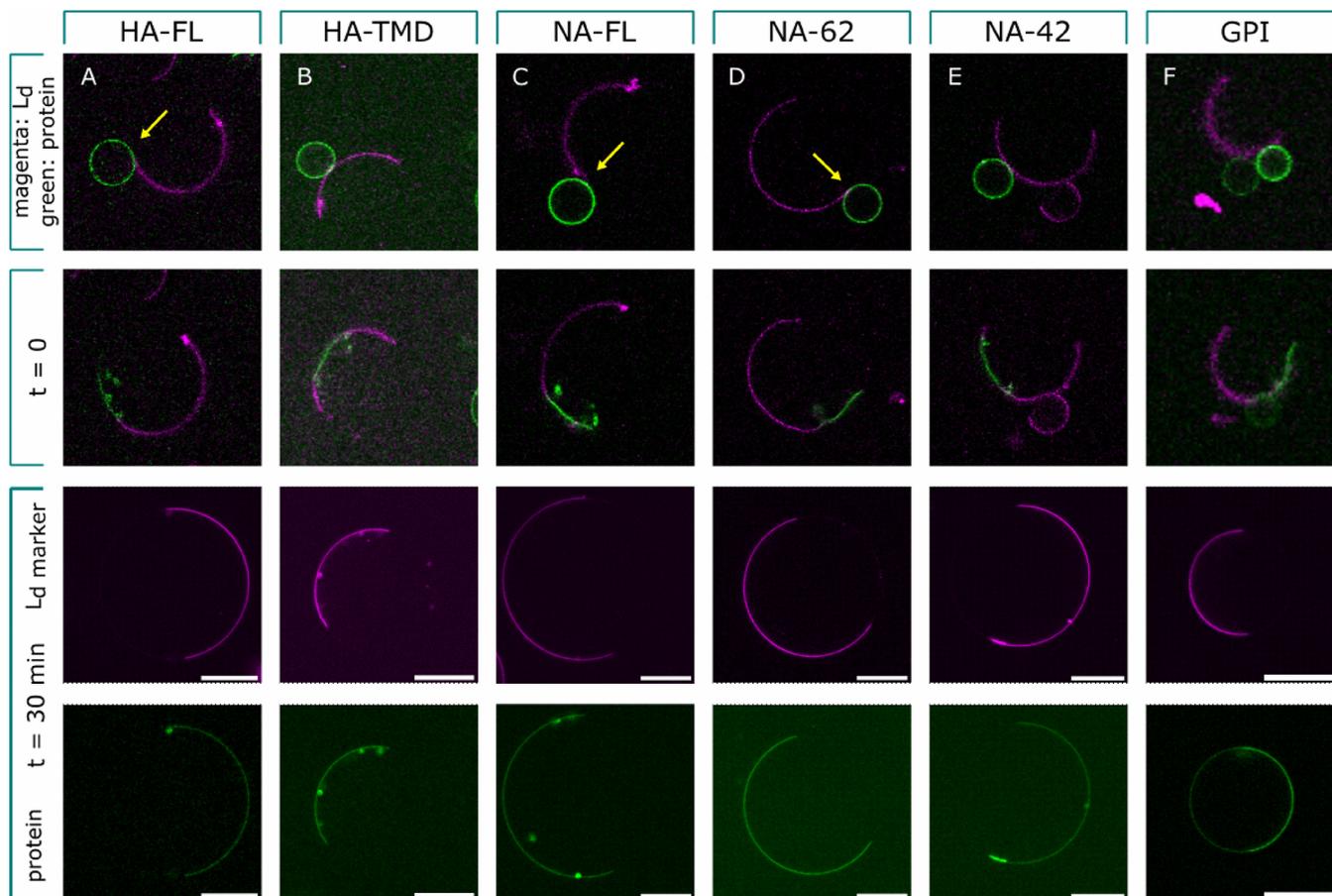
methods for studying rafts are based on biochemical, biophysical, computational, and analytical tools.<sup>31</sup> Biochemical tools use detergents to solubilize membrane lipids and proteins<sup>32</sup> which do not properly reflect the native molecular structure and organization of rafts. Biophysical tools are mainly based on model membranes,<sup>33</sup> including giant unilamellar vesicles (GUVs), into which membrane proteins can be reconstituted using biochemical protocols.<sup>34,35</sup> Despite the high resolution provided by some assays, most analytical tools require sample preparation involving tedious procedures<sup>36</sup> which can potentially influence the protein localization in model membranes.

Membrane proteins from influenza virus have been studied using cell-derived giant plasma membrane vesicles (GPMVs) which can exhibit large scale phase segregation only at very low temperatures ( $\sim 5$  °C) and by addition of the cross-linking agent cholera toxin B.<sup>34,37</sup> These studies have shown that NA and HA do partition into the more disordered lipid phase which is somewhat conflicting with the idea that viruses bud from cholesterol enriched domains. These findings have opened up new questions on how virus budding and plasma membrane structure are related, thus raising the possibility that raft association of virus proteins could be mediated through interactions between different proteins. However, the low temperature, and use of a toxin to trigger phase separation, raises concerns on whether this system sufficiently reflects the cellular plasma membrane.

Here, we present a general assay which can address the localization of transmembrane proteins in phase-separated

membranes at a temperature set by the phase transition temperature of the lipid mixture and without the need of any chemical cross-linking agent. Phase-separated GUVs are fused with GPMVs containing the protein of interest in the correct orientation. The fused hybrid vesicle contains phase separated domains which allow us to study the localization of membrane protein phase affinity at physiologically relevant condition. We demonstrate the simplicity and efficacy of the method by investigating the phase affinity of influenza A virus membrane proteins and several chimeras containing specific domains of these proteins. Fusion of the vesicles is accomplished using thermoplasmonics and optical trapping.<sup>38–40</sup> This generic method can be applied to any membrane protein expressed in cells and does not require protein purification. Finally, it also holds the potential for studying interactions between either membrane proteins or between membrane and peripheral membrane binding proteins in a controlled manner under physiological conditions.

Chimeric fluorescent proteins of interest were transiently expressed in HEK293T cells to obtain the membrane proteins under physiological conditions. Following expression, a vesiculating reagent is added to the cells which triggers cells to detach part of their plasma membrane, containing the associated proteins, as giant plasma membrane vesicles (GPMVs), also known as blebs. The produced GPMVs hold the protein of interest in correct orientation with intact functionality.<sup>37</sup> To transfer the membrane proteins to a model membrane, we mix the GPMVs with phase-separated GUVs in a cell culture dish (Figures 1A and S1) at room temperature



**Figure 2.** Phase preference of influenza HA, NA, and a GPI anchor control. The GPMVs extracted from the plasma membrane accommodate the GFP-tagged protein of interest in their membranes. The disordered phase of the GUVs is marked with the  $L_d$  marker 18:1 Liss Rhodamine PE that exclusively partitions into the disordered region. The first row shows the positions where the fusion happens (yellow arrows) at the ordered-disordered phase interface. At time zero ( $t = 0$ ), the protein is mainly found near the site of fusion. The phase preference of all proteins was evaluated after 30 min (lowest two rows). (A) Full-length HA protein and (B) its transmembrane domain colocalize with the liquid disordered phase. (C) Full-length NA and two C-terminally truncated variants (NA- $\Delta$ head) (D) and (NA-TMD) (E) distribute into the disordered region. (F) GPI anchored GFP used as the positive control for liquid order phase partitioning.<sup>64</sup> The images have been adjusted in brightness and contrast for better visualization. Scale bars are 15  $\mu\text{m}$ .

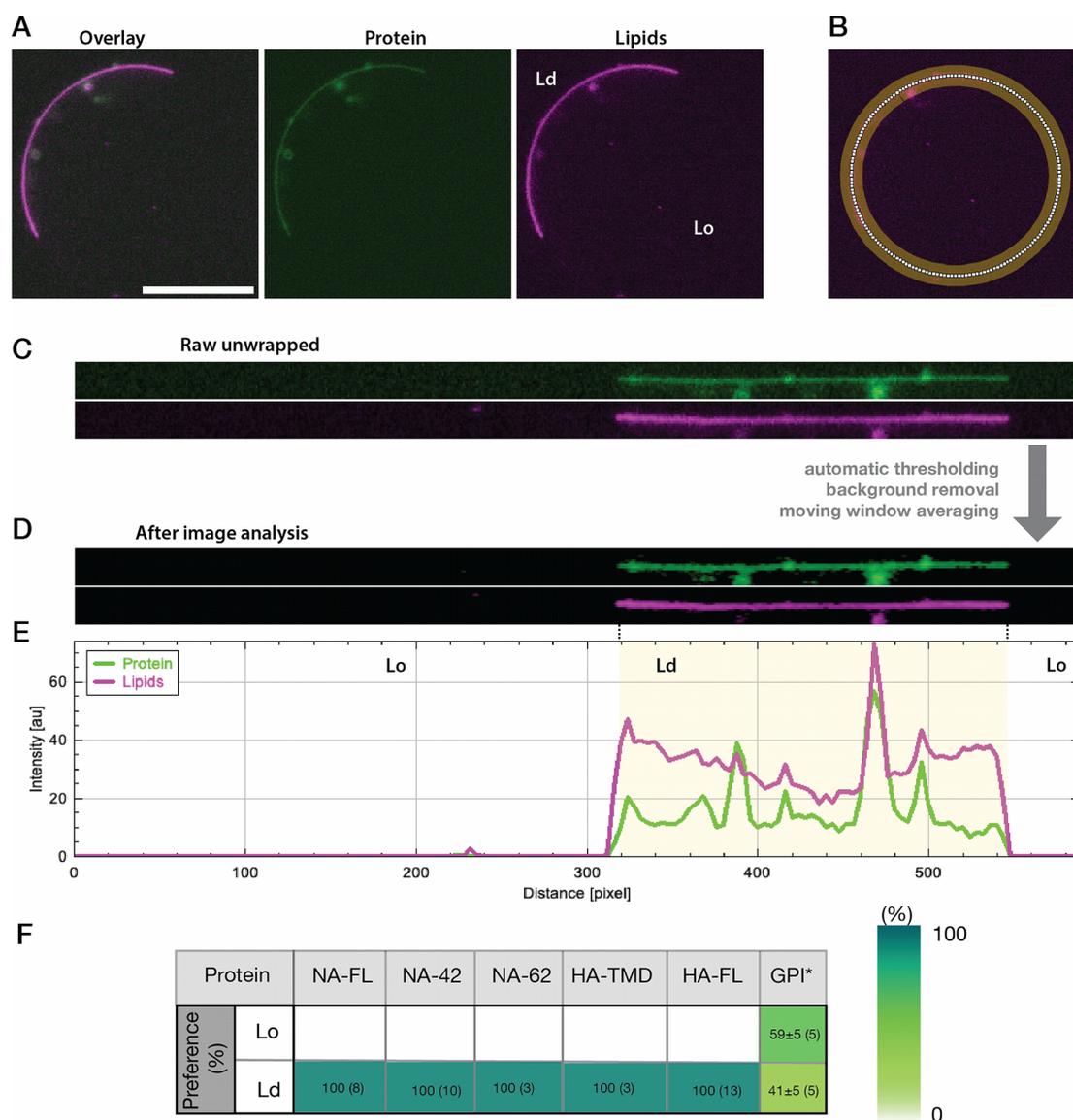
which ranged from 20 to 25  $^{\circ}\text{C}$ . The GUVs vary in size with a diameter between 10 and 80  $\mu\text{m}$  and are composed of saturated fatty acids plus cholesterol and unsaturated lipids. The stoichiometry of lipids and cholesterol (Table S1) allows formation of a stable liquid-ordered phase ( $L_o$ , raft resembled domain) and liquid-disordered phase ( $L_d$ , nonraft resembled domain), respectively, at room temperature ( $T = 22$   $^{\circ}\text{C}$ ).

Because the GPMVs also differ in size, variations in the GUV's diameter enable us to select GUV–GPMVs of comparable size for a successful fusion (Figure 1B). The NIR laser ( $\lambda = 1064$  nm) is then used to grab and position the GPMV of choice in close proximity to the selected GUV. We have included more details in the Supporting Information on the time scale of fusion, the protein partitioning dependence on relative vesicle sizes, and the fusion to different phases in the GUV (see Figures S2 and S3).

The 150 nm streptavidin conjugated gold nanoshells (AuNSs) are bound to biotinylated lipids in the GUV membrane. When the close contact between the GPMV and the GUV is established, the focus of the optical trap is positioned at the contact point of the two apposing membranes. The gold nanoparticles are mobile on the fluid GUV membrane and are pulled into the optical focus (Figure

1C). Irradiation of the AuNS by the NIR laser, operated at  $3.04 \times 10^{10}$   $\text{W m}^{-2}$  at the sample, produces highly localized heat (on the order of  $\Delta T \sim 200$  K)<sup>40</sup> which is sufficient to transiently open the two apposing membranes and, thereby, fuse the vesicles together to form a hybrid vesicle (Figure 1C). AuNSs produce a thermal pulse when entering the optical focus and subsequently escape the focus or become structurally degraded.<sup>41</sup> The membrane associated proteins are delivered to the phase-separated model membrane and are allowed to mix and partition to the phase of their preference (Figure 1D).

We examined the influenza membrane proteins HA and NA for which the phase affinity has been highly disputed in the literature.<sup>15,42–45</sup> Association of viral proteins with raft domains has been difficult to discern, mainly because raft domains are highly dynamic and of submicrometer size, thus making them difficult to be directly visualized in living cells. While HA has been reported to associate with rafts by cell surface analysis,<sup>46</sup> it has also been shown to partition into nonraft domains of model membranes.<sup>34</sup> However, the lipid preference of HA may be determined by the presence or absence of a palmitoyl anchor which could be responsible for recruiting it to ordered and raft-like phases.<sup>47</sup> Here, we used an engineered plasmid to transiently transfect HEK293T cells to



**Figure 3.** Quantification of protein partitioning. (A) Raw data from fusion experiment. GFP-tagged hemagglutinin transmembrane domain, abbreviated as HA-TMD (in green), and membrane (magenta) channels are overlaid. Scale bar is 15  $\mu\text{m}$ . (B) Image exemplifying the selection of the vesicle by the image processing workflow. (C) Raw, unwrapped vesicle fluorescent channels. (D) Unwrapped vesicles after processing with image processing workflow. (E) Fluorescent intensity from the unwrapped vesicles from (D).  $L_d$  region is highlighted in yellow. (F) Lipid order preference from each of the proteins used in this project. We quantified the percentage of protein fluorescence signal coming from  $L_o$  and  $L_d$  lipid regions. The number in parentheses indicates the number of fusions performed for each type of protein. \* denotes  $L_o$  preference.

express a full-length HA (HA-FL) which has its C-terminal tagged with a green fluorescent protein (GFP) and is predominantly found to form trimers.<sup>48</sup> Also, we engineered a plasmid encoding for HA transmembrane domain with its N-terminal tagged with GFP which is additionally found to form both monomers and dimers.<sup>48</sup> We used plasmids encoding full length neuraminidase (NA-FL) and two variants that contained the NA-transmembrane domain and were C-terminally truncated at residue 42 (NA-42) or 62 (NA-62), removing the large head domain and leaving different portions of the stalk (Figure S4).<sup>37,49</sup> A similar strategy was followed for the GPI linked GFP control (Figure S5).

Similarly to what has been reported previously for recombinant HA reconstituted into phase-segregated vesicles,<sup>50</sup> we find that HA segregates into  $L_d$  domains in our hybrid vesicles after fusion (Figure 2A). Therefore,

contrary to the widely accepted theory of influenza virus assembly,<sup>15</sup> HA expressed alone in HEK293T cells does not concentrate in cholesterol enriched lipid domains.

To exclude the possibility that the GFP label attached to the intracellular part of the HA could interfere with the palmitoylation sites, we tested the phase preference of the HA transmembrane domain (HA-TMD) with the ectodomain tagged with a GFP. However, as shown in Figure 2B, the HA-TMD also exhibited complete partitioning into the disordered phase, thus ruling out any interfering effect of the GFP label.

Influenza NA (NA-FL) displayed a preference for disordered domains, and this was confirmed by the observation of similar results using NA constructs without the enzymatic head domain (NA-62) and without both the head domain and majority of the stalk region (NA-42), as shown in Figures 2C–

E and S5. We note that NA-42 assembles into dimers within the membrane.

As a control, we investigated the partitioning of a GPI anchored GFP which has been found to localize preferentially into liquid ordered phases in GPMVs.<sup>51</sup> As expected, GPI predominantly localized into liquid ordered regions of the hybrid vesicles as shown in Figure 2F.

A total of 37 fusion experiments with varying protein constructs confirm partitioning of the proteins into the  $L_d$  phase (Figure 3). Quantification of partition coefficients was not possible due to complete partitioning of all proteins apart from the GPI control. As shown in Figures 3 and S6, all ordered phases (apart from the GPI experiment) contained no fluorescent signal after subtraction of the image background. Hence, we conclude that within the sensitivity of our imaging system, both HA and NA exhibit complete partitioning into  $L_d$  phases whereas for the GPI control we found a slight  $L_o$  preference equivalent to a partition coefficient  $K_{p,raft} = 1.4$ .  $K_{p,raft}$  was calculated as in ref 51 where  $K_{p,raft} = \text{Int}_{L_o}/\text{Int}_{L_d}$ .

Our results show that both HA and NA do indeed localize into disordered domains, and it remains uncertain whether other effects like protein–protein interactions, between different types of virus proteins, could change this phase preference.

Reports on partitioning of virus proteins into  $L_o$  or  $L_d$  phases have yielded conflicting results. Early reports found that the transmembrane domain of HA interacted with sphingolipids and cholesterol as was found by isolating detergent-insoluble fractions from the plasma membrane;<sup>52</sup> however, the reliability of the detergent-resistant method has been questioned.<sup>53,54</sup> Using immuno-electron microscopy together with gold labeling, HA was shown to form nanoclusters in fixed cellular plasma membranes. However, no association between HA and rafts was found because it still clustered after depletion of the two raft constituents glycosphingolipids and cholesterol.<sup>44</sup> These methods entail procedures that capture raft associated proteins in a single time frame and not in a real-time manner. Other studies have found that HA partitions into raft phases only when a palmitoylation site is present.<sup>34</sup> Palmitoylation of proteins has been found to be a major factor for recruiting integral membrane proteins to ordered domains.<sup>2,11,29,47</sup> However, this ordered phase recruitment of HA, mediated by the palmitoylation site, is a mechanism which is not supported by our data. One scenario is that the cytoplasmic GFP label could interfere with the function of the palmitoylation anchor for the HA-FL. However, the HA-TMD which has its GFP label on the ectodomain still partitioned exclusively into the disordered phase. Therefore, we conclude that the palmitoylation site in HA is not sufficient to direct localization of the HA protein into ordered phases of our hybrid vesicles.

Other factors which could influence protein partitioning are the hydrophobic match of the transmembrane domains with the thickness of the hydrophobic interior of the membrane<sup>55</sup> and the presence of actin. The cortical actin structure has been found to organize membrane proteins into clusters<sup>11,27</sup> and even affect the formation of  $L_o$  and  $L_d$  phases,<sup>43</sup> thus highlighting the fact that membrane organization alone is not the sole factor determining membrane protein distribution in living cells. Our results, however, show how protein partitioning behaves in the absence of actin because GPMVs, used here for the formation of hybrid vesicles, are known to be devoid of assembled cytoskeletal structure.<sup>56</sup> Interactions

between proteins is another factor which could influence protein partitioning, as shown recently in ref 57, where a complex interaction was reported between influenza A membrane proteins during viral budding. These findings, together with our results, suggest that there could be more complex interactions at play during viral budding than previously anticipated.<sup>58</sup>

Delivering plasma membrane proteins to GUVs by this method provides several advantages. The control of the fusion site allowed us to test for protein partitioning after fusion into (i) the  $L_d$  phase, (ii) the  $L_o$  phase, or (iii) the  $L_d/L_o$  phase interface. An overview of protein partitioning from these three fusion sites is given in Figure S2C and shows that protein partitioning does not depend on where fusion takes place on the GUV. In addition, because the fused GPMVs are derived from transfected cells, they contain the lipid and protein diversity of the plasma membrane, although in a diluted version. Importantly, the final hybrid vesicle contains several types of proteins correctly oriented, as seen from a biological perspective, with ectodomains pointing outward.

The GPMV/GUV hybrid vesicle assay adds another biologically relevant model system to be used for studying proteins in membranes, and the question remains how the membrane in the hybrid vesicles is related to the phases in more studied GPMVs and GUVs.  $L_o/L_d$  phases in GUVs form due to interactions between cholesterol and the saturated acyl chains, and this interaction is known to form phases with significantly different lipid order as quantified by measuring the hydration level or, equivalently, the generalized polarization ( $G_p$  value) using environmentally sensitive dyes like Laurdan.<sup>59</sup> However, this large difference between  $L_o$  and  $L_d$  phases is known to be much attenuated in both cooled GPMVs and osmotically induced plasma membrane spheres (PMS induced at 37 °C) which should closely mimic the plasma membrane of living cells.<sup>60</sup> Importantly, the hybrid vesicles in our work would mimic the plasma membrane to a higher degree than GUVs, but the phases observed in these hybrid vesicles must be somewhat affected by the presence of lipids and cholesterol from the GUV. Our hybrid vesicles contain both glycans, various proteins, and a host of lipids which “smooth” the phase differences to become more cell-like with small differences in  $G_p$  value as measured by Laurdan in GPMV.<sup>60</sup>

The thermal damage to lipids can be neglected due to the fact that the heating region surrounding the heated nanoparticle is highly localized and transient. Our previous and extensive work on the plasmonic heating of these nanoparticles clearly show that the high temperatures ( $\Delta T > 100$  °C) are only located within a distance of ca. 100 nm from the particle surface which has been confirmed in both experiments and simulations.<sup>40,61</sup> Moreover, the time scale of fusion is on the order of a second.<sup>62</sup> Lipids in freestanding lipid bilayers and in GPMVs have a diffusion constant of  $D \sim 5 \mu\text{m}^2/\text{s}$  and for neuraminidase proteins  $D \sim 1 \mu\text{m}^2/\text{s}$ ,<sup>37</sup> and hence only limited mixing between the locally heated area and the rest of the large GUV/GPMVs takes place within this short time. Hence, we conclude that only minor protein and lipid damage can be expected from this method simply due to the short time a small fraction of the lipids are exposed to the heating.

We present a new technique that allows for effective transfer of membrane proteins to a phase-segregated model membrane system in which phase partitioning can be observed at physiological conditions without the addition of chemical and cross-linking reagents. With this method we demonstrate

that the viral spike proteins HA and NA partition predominantly into the liquid disordered phase. Further studies will reveal how coexistence of other proteins from influenza A virus embedded in a model membrane affect their mutual preference for a specific lipid phase. An extremely interesting future perspective of the current method is the possibility of combinatorial selection of proteins to be added sequentially to a hybrid GUV/GPMV model membrane. Influenza virus proteins HA, NA, M1, and M2 are populating the virus envelope, and our assay can be employed to systematically investigate possible interprotein interactions which could change their phase affinity and even other membrane remodeling behavior that these proteins could be responsible for. Also, the introduction of cytoskeletal structures into hybrid vesicles is straightforward using our method. This can be achieved by fusion of GUVs containing actin monomers and actin-membrane linkers to GPMVs which contain the cytosolic actin polymerization factors from the donor cell. In general, our method allows for generic investigation of how single proteins, and mixtures of proteins, collectively organize in heterogeneous membranes which will advance our understanding of protein function in complex biological membranes.

## ■ ASSOCIATED CONTENT

### Data Availability Statement

The data that support the findings of this study are openly available in Zenodo at [10.5281/zenodo.7577427](https://doi.org/10.5281/zenodo.7577427) in ref 63.

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.nanolett.3c00371>.

Data analysis, design and preparation of plasmids, transfection and fusion protocols, GPMV isolation, GUV formation, and additional figures (PDF)

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## Author Contributions

G.M.-P. and M.R.A. contributed equally to this work.

## Notes

The authors declare no competing financial interest.

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