Engineering Planar Gram-Negative Outer Membrane Mimics Using Bacterial Outer Membrane Vesicles

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Abstract:

Antibiotic resistance is a major challenge in modern medicine. The unique double membrane structure of gram-negative bacteria limits the efficacy of many existing antibiotics and adds complexity to antibiotic development by limiting transport of antibiotics to the bacterial cytosol. New methods to mimic this barrier would enable the high-throughput studies for antibiotic development. In this study, we introduce an innovative approach to modify outer membrane vesicles (OMVs) from *Aggregatibacter actinomycetemcomitans*, to generate planar supported lipid bilayer membranes. Our method first involves the incorporation of minimal synthetic lipids into OMVs using a rapid freeze-thaw technique to form outer membrane hybrid vesicles (OM-Hybrids). Subsequently, these OM-Hybrids can spontaneously rupture when in contact with SiO_2 surfaces to form a planar outer membrane supported bilayer (OM-SB). We assessed the formation of OM-Hybrids using dynamic light scattering and a fluorescence quenching assay. To comprehensively analyze the formation of OM-SBs from OM-Hybrids we used quartz crystal microbalance with dissipation (QCM-D) and fluorescence recovery after photobleaching (FRAP). Additionally, we conducted assays to detect surface-associated DNA on OM-SBs, a characteristic property often observed in OMVs. These findings emphasize the capability of our platform to produce planar surfaces of bacterial outer membranes, which in turn, could function as a valuable tool for streamlining the development of antibiotics.

Introduction:

The escalating challenge of antibiotic resistance in modern healthcare necessitates urgent attention and innovative solutions to preserve the efficacy of antimicrobial treatment[1,2]. To address this pressing issue, there is a need to explore new methods for antibiotic development and testing. In light of the growing resistance to conventional antibiotics, antimicrobial peptides (AMPs) have gained prominence as an alternative[3–5]. They target bacterial membranes, reducing the potential for resistance to develop. However, evaluating the interaction of AMPs with bacterial membranes presents a considerable challenge, as it involves creating a membrane model system that faithfully replicates bacteria while enabling in vitro testing.

Gram-negative bacteria display distinct cellular architecture characterized by a double membrane structure consisting of inner and outer membranes[6]. These membranes encase a relatively thin layer of peptidoglycan. The outer membrane in gram-negative bacteria exhibits heterogeneity, with the outer leaflet comprising lipopolysaccharide (LPS) and outer membrane proteins, while the inner leaflet is predominantly composed of phospholipids[6]. For antibiotics, specifically AMPs, to be successful for gram-negative bacteria treatment, it must first interact with or be permeable through the other membrane, therefore any changes to the outer membrane can lead to resistance[7,8]

Having a planar surface that mimics the bacterial outer membrane offers multiple advantages. Firstly, it provides a valuable tool for assessing interactions between potential antibiotics, such as AMPs, and the bacterial membrane using a variety of analytical techniques, such as surface-based biosensors atomic force microscopy[9,10], fluorescence microscopy[11], and quartz crystal microbalance[12]. In recent years, the utilization of synthetic lipids to create planar bilayers has emerged as a significant biotechnological advancement, and it has also been employed in the past for testing the effect of AMPs on gram-negative bacteria, where bacterial lipids with known composition were mixed to prepare vesicles[13–15]. However, synthetic lipid vesicles have several disadvantages, including that their composition is not an exact mimic of the outer membrane. In addition, these mimics do not contain proteins, which play a prevalent role in the outer membrane. An alternative to using synthetic lipids for vesicle production is to extract lipids directly from the bacteria[14,16,17]. However, a disadvantage of this method is that when bacterial lipids are extracted, there will be lipids present from both the outer and inner membranes of the bacteria, and the origin of the lipids cannot be precisely determined.

Gram-negative bacteria exhibit a fascinating phenomenon in which they bud their outer membranes to generate structures known as outer membrane vesicles (OMVs)[18,19]. These OMVs are crucial for bacterial

communication and cargo transport. Furthermore, they play a significant role in pathogenicity as they can carry and deliver toxins to host cells[20–22]. Because OMVs originate from the bacterial outer membrane, they provide valuable insights into the composition of the outer membrane, including details about LPS, lipid composition, and proteins[23,24]. Given that OMVs closely mimic the outer membrane of their parent bacteria, they present a promising opportunity to serve as bacterial membrane mimics. However, OMVs are nanometer sized spheres, which unlike liposomes, do not rupture on glass or silicon dioxide surfaces to form a planar supported lipid bilayer (SLB). This phenomenon can be attributed to the presence of anionic lipids, such as phosphatidylglycerol and LPS in OMVs, which hinders the electrostatic interactions between OMVs and glass necessary for rupture to occur.

Previously Daniel and coworkers reported OMV-based supported lipid bilayers[25,26]. In this method, the OMVs were packed in a high density on a surface, followed by the addition of synthetic liposomes to induce the rupture of OMVs. While this method does allow for the formation of a surface incorporating the OMVs, it does have limitations, including that it requires the OMVs to be packed in a dense manner, requiring a high concentration of OMVs. In addition, upwards of 2 mg/mL of synthetic lipid was used to induce the rupture of OMVs, which dilutes the OMV components in the surface. Additionally, there is no direct measure of the synthetic lipid and OMV interaction.

Our goal in this work was to create hybrid outer membrane vesicles (OM-Hybrids) that can spontaneously rupture on a silica dioxide surface to create outer membrane-supported bilayers (OM-SB). Previously, OMVs were shown to fuse with eukaryotic cells to form prokaryotic-eukaryotic hybrids for drug delivery using methods like sonication and extrusion[27–31]. Additionally, Pace et al. described a method involving cell lysis to create vesicles, followed by their fusion with synthetic liposomes using sonication, leading to their ability for spontaneous rupture and supported bilayer formation[32]. While this method is effective, our objective was to investigate the feasibility of using freeze-thaw as an alternative approach to create OM-Hybrids. Previously, rapid freeze-thaw has been reported as a suitable technique for lipid-lipid mixing[33]. Moreover, freeze-thaw has demonstrated success in generating exosome-liposome hybrids, which can be utilized for drug delivery[34,35]. Freeze-thaw has previously been reported to successfully load OMVs with molecular cargo[36–39]. This suggests that freeze-thaw treatment effectively induces lipid disruption and reformation within OMVs, a property that can be harnessed for OM-Hybrid development, which can subsequently be used to form OM-SBs.

As a proof of concept, we utilized OMVs produced by *Aggregatibacter actinomycetemcomitans*, a gram-negative bacterium associated with severe periodontal diseases[40]. Treatment of *A.*

actinomycetemcomitans infections generally involves scaling and root planning followed by systemic antibiotics[41,42]. However, there have been numerous reports of its resistance to commonly used antibiotics for treatment[43,44]. As part of its virulence, *A. actinomycetemcomitans* produces leukotoxin (LtxA), which is secreted into the extracellular milieu as a free protein and is also released in association with OMVs, where it resides on the surface along with surface-associated DNA [45–47]. This highlights the critical role of OMVs in pathogenesis, which is why we seek to utilize them, given their relevance in the disease process. For the synthesis of OM-Hybrids, we utilized liposomes consisting of 99.5 mole % phosphatidylcholine (POPC) and 0.5 mole % polyethylene glycol attached to a phosphatidylethanolamine lipid tail group (PEG-PE). The PEG moiety adds an extra cushion to the resultant OM-SB that prevents the bilayer from touching the surface, which prevents proteins from becoming pinned on the surface[32,48] .

We assessed the OM-hybrid formation through techniques such as fluorescence quenching and dynamic light scattering (DLS), providing insights into their characteristics. We also determined the lipid diffusion coefficient of the OM-SB through fluorescent recovery after photobleaching (FRAP) and observed that OM-SB exhibited a lower diffusion coefficient compared to the POPC/PEG-PE supported lipid bilayer (POPC/PEG-PE SLB). Additionally, we utilized quartz-crystal microbalance with dissipation monitoring (QCM-D) to assess OM-SB formation from OM-Hybrids. Finally, we investigated the presence of surface-associated DNA on these OM-SBs, demonstrating that our hybrid formation method preserves the integrity of the OMVs and their surface-associated components. This method exhibits versatility, facilitating the generation of planar membranes possessing bacterial biomolecules while maintaining a minimal incorporation of synthetic lipid components. Furthermore, its gentle nature ensures the preservation of critical outer membrane constituents.

Experimental Section:

***Reagents and chemicals*:** The following lipids used in this study were bought from Avanti polar lipids: 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (ammonium salt) (PEG-PE). Octadecyl rhodamine B Chloride (R18), DiI, and DiO membrane labels were bought from Invitrogen. Triton X-100 was purchased from Sigma-Aldrich. Polydimethylsiloxane (PDMS) was purchased from Dow. Oxazole yellow (nucleic acid stain) was purchased from Biotium. Anti-OmpA rabbit polyclonal antibody was purchased from Antibody Research Corporation. A secondary antibody with an Alexa-488 label was purchased from Abcam. All experiments were performed in Tris buffer composed of 150 mM NaCl and 10 mM Tris-base (pH 7.0).

Liposome preparation: A molar ratio of 99.5% POPC to 0.5% PEG-PE was dissolved in chloroform. The resulting solution was mixed within a glass vial, followed by the evaporation of the solvent to yield thin lipid films. The lipid films were rehydrated in Tris buffer with a final lipid concentration of 1 mg/mL, followed by gentle vortexing and bath sonication for 10 minutes. The resulting liposomes were extruded through a 50 nm pore diameter polycarbonate membrane (Cytiva Life Sciences) using a Mini-Extruder (Avanti Polar Lipids) with 23 passes.

Purification of A. actinomycetemcomitans OMVs: *A. actinomycetemcomitans* strain JP2 was raised in trypticase soy broth (30 g/L, BD Biosciences) and yeast extract (6 g/L, BD Biosciences) with the addition of 0.4% sodium bicarbonate (Fisher Scientific), 0.8% dextrose (BD Biosciences), 5 μg/mL vancomycin (Sigma-Aldrich), and 75 μg/mL bacitracin (Sigma-Aldrich). An initial culture was grown at 37 °C for 16 hours in a candle jar, then used to inoculate a larger culture that was allowed to grow for 24 hours at 37 °C. The bacteria were centrifuged once for 10 minutes at 10,000 × g, twice for 5 minutes at the same speed, and then filtered through a 0.45 μm filter. Following a 30-minute ultracentrifugation at 105,000 × g, the pellet containing the OMVs was resuspended in PBS (pH 7.4) and ultracentrifuged once more. Lastly, the final pellet was resuspended in PBS.

OM-Hybrid preparation: Equal volumes of 0.2 mg/mL POPC/PEG-PE liposomes were mixed with OMVs that had a total lipid concentration of 4.5 mM. The solution was rapidly frozen at -196 ℃ using liquid nitrogen and subsequently thawed at 45 ℃ using a hot water bath. The freeze-thaw cycle was repeated ten times, and at the end of the tenth cycle, the resultant OM-Hybrid vesicles were left in the water bath for 10 minutes.

Dynamic light scattering (DLS): DLS analysis was used to measure the diameters of liposomes, OM-Hybrids, and OMVs. Both OMVs alone and liposomes alone underwent ten freeze-thaw cycles to make a direct comparison with OM-Hybrid production. Liposomes were diluted in Tris buffer to a final concentration of 0.25 mg/mL and OMVs and OM-Hybrids were diluted in Tris buffer with a dilution factor of 1:100. We used an ALV/CGS-3 Compact Goniometer System to make DLS measurements. At a wavelength of 623.8 nm and a scattering angle of 90°, data were gathered for 120 seconds in triplicate. The ALV software used a number-weighted regularized fit with an allowed membrane thickness (r*) of 5 nm to calculate the size distribution. .

Lipid mixing study: 0.2 mg/mL of POPC/PEG-PE liposomes containing 10 mole % R18 were prepared and mixed with non-fluorescent OMVs (4.5 mM lipid concentration) in equal volume, followed

by rapid freeze thaw, as described above. The samples were then diluted 1:101 and added to quartz cuvette for fluorescence spectroscopy. Fluorescence emission spectra were collected using a Fluorolog TCSPC fluorometer (Horiba Scientific). The excitation was held constant at 500 nm while the emission was collected from 530 – 700 nm.

Fluorescence recovery after photobleaching and diffusion coefficient calculations: Glass coverslips were cleaned first with isopropyl alcohol, then submerged in an aqueous 2% sodium dodecyl sulfate (SDS) solution for at least 30 minutes. The glass coverslips were thoroughly rinsed with milliQ H_2O and then exposed to UV-Ozone (UV/Ozone ProCleaner Plus, BioForce Nanosciences) treatment for a duration of 10 minutes. To create small wells, first an ultra-flat PDMS slab was prepared, and the well was created within the PDMS slab using a 6 mm biopsy hole punch. The PDMS well was plasma treated for 90 seconds and then attached to the clean glass coverslip. OM-Hybrids labeled with DiI or 0.2 mg/mL of POPC/PEG-PE liposomes labeled with DiI were incubated on the glass coverslip for 1 hour and, then the resulting planar bilayers were washed with Tris buffer. The bilayers were imaged using an inverted microscope (Eclipse Ti, Nikon) equipped with a 100× oil immersion objective with a 1.49 numerical aperture. Fluorescence was excited using an LED light engine (Aura II, Lumencor) and appropriate filter cubes. Images were captured with a 2048 × 2048 pixel sCMOS camera (Orca Flash 4.0 v2, Hamamatsu). The planar bilayers were photobleached using a 405 nm laser (50 mW) pulse for 1 seconds and fluorescence recovery was captured at 1 seconds intervals for 60 seconds.

Quartz crystal microbalance: A QSense Explorer E1 QCM-D instrument from Biolin Scientific, equipped with a 5 MHz AT-cut SiO_2-coated quartz crystal (Nanoscience Instruments), served as the platform for conducting QCM-D studies. In the flow-cell, OM-SB hybrids, liposomes at a concentration of 0.20 mg/mL, or OMVs with a lipid concentration of 2.45 mM were introduced, flowing at a rate of 100 µL/min. While the 1st, 3rd, 5th, 7th, 9th, and 11th overtones were monitored, only the data corresponding to the 3rd harmonic is reported. The flow-cell temperature was held constant at 23 °C.

OM-SB DNA assay: OM-SB and POPC/PEG-PE bilayers were formed on clean glass coverslips as described above. After removing any extra vesicles, a PDMS well was filled with a 1:1000 dilution of YO-PRO-1 (Biotium), which was then incubated for an hour. Bilayers were observed using an inverted microscope (Nikon Eclipse Ti) fitted with a 100× oil immersion objective with a 1.49 numerical aperture after the extra dye had been removed. A TRITC and FITC (Chroma), and an LED light engine (Aura II, Lumencor) were utilized to excite and observe the fluorescence. A 2048 × 2048 pixel sCMOS camera (Orca Flash 4.0 v2, Hamamatsu) was used to collect images. ImageJ (NIH) was used to analyze and overlay images.

Results and Discussions:

Formation of OM-Hybrids for OM-SB formation: The establishment of planar SLBs through the rupture of synthetic liposomes on glass and SiO_2 surfaces is a common technique widely used for the creation of planar bilayer surfaces[49]. In general, liposomes composed of zwitterionic phospholipids, such as POPC, readily adsorb and rupture to form SLBs on glass and SiO_2 surfaces. OMVs released by gram-negative bacteria exhibit the opposite behavior—they do not rupture upon contact with glass surfaces; instead, they sparsely adhere to the glass and remain intact **(Figure S1)**. In order to surmount this challenge, our aim was to engineer hybrid vesicles that would be amenable to planarization by vesicle rupture. DiI labeled OMVs were mixed with non-fluorescent liposomes (99.5% POPC, 0.5% PEG-PE) in an equal volume. The mixture was rapidly frozen using liquid nitrogen, followed by a subsequent thawing process at 45°C. This process was repeated 10 times to ensure a complete lipid mixing **(Figure 1).** After the completion of the tenth cycle, the OM-Hybrids were immersed in a warm water bath at 45°C and subsequently exposed to a clean glass coverslip to form a planar OM-SB.

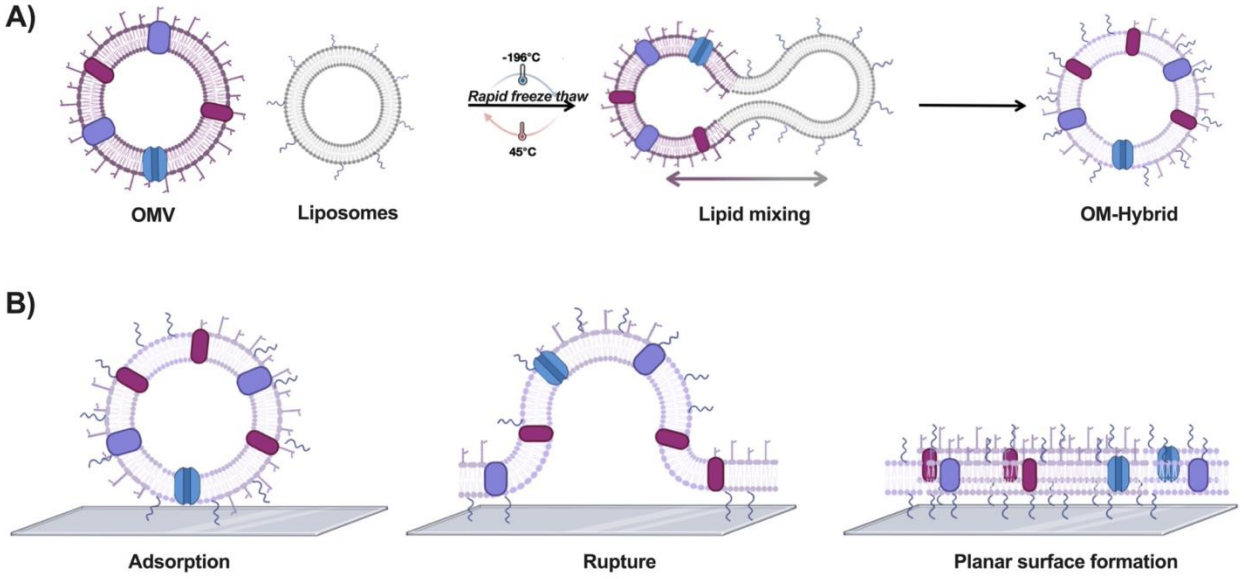

Figure 1: Schematic representation of the process: (A) Equal volumes of OMVs and POPC/PEG-PE liposomes are mixed and subjected to rapid freeze thaw (FT)- treatment. The FT-treatment induced lipid breaking and reformation, which results in lipid mixing and formation of OM-Hybrids. **(B)** OM-Hybrids adhere to the clean glass surface, initiating interactions that lead to their rupture and the formation of a planar surface.

Lipid mixing between POPC/PEG-PE and A. actinomycetemcomitans OMVs: To investigate the interaction between liposomes and OMVs and the formation of OM-Hybrids, we employed the R18 fluorescent dye. This dye is recognized for its self-quenching behavior at elevated concentrations and has

previously been demonstrated as effective for studying OMV-OMV fusion[50]. The experimental strategy involved creating POPC/PEG-PE liposomes that contained 10 mole % of R18, and subsequently monitoring the fluorescence enhancement upon freeze-thaw treatment with non-fluorescent OMVs. If lipid mixing was occurring, an increase in fluorescence was expected. This phenomenon could be attributed to the dispersion of dye molecules away from each other, facilitated by the increased non-labeled OMV component, thus leading to heightened fluorescence levels. Initially, as a validation of the concept, we exposed R18-labeled POPC/PEG-PE liposomes to a treatment with 0.1% Triton. Triton lyses the liposomes, liberating the R18 molecules and resulting in an increase in observed fluorescence **(Figure S2).** We noted a significant 6.3-fold enhancement in dye emission following the complete lysis of liposomes and the subsequent release of the R18 fluorescent dye. This observation demonstrates that this method holds promise for investigating lipid mixing processes.

Equal volumes of R18 labeled POPC/PEG-PE liposomes and OMVs were mixed and subjected to either five or ten freeze-thaw cycles. After subjecting the POPC/PEG-PE liposomes and OMVs to five freeze-thaw cycles, we observed a modest 1.2-fold increase in fluorescence compared to freeze-thawed POPC/PEG-PE liposomes alone, which did not reach statistical significance **(Figure 2)**. However, upon performing ten freeze-thaw cycles, we noted a statistically significant 1.5-fold increase in fluorescence. Consequently, we chose to implement ten freeze-thaw cycles in our protocol to ensure thorough membrane mixing. Furthermore, we sought to determine if the increase in fluorescence was a result of freeze-thaw treatment. To investigate this, equal volumes of R18 labeled POPC/PEG-PE liposomes with non-labeled OMV were mixed, but were examined without the freeze-thaw (FT) treatment. In this case, we observed a non-significant change in fluorescence intensity between liposomes and the non-treated samples. The mean

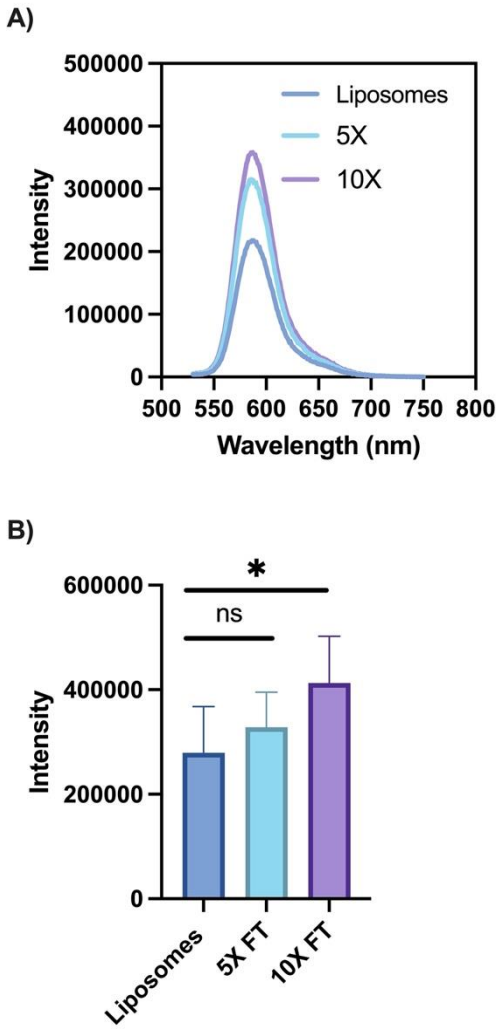

Figure 2: Quantifying lipid mixing through monitoring R18 fluorescence increase: (A) Representative spectra of the fluorescence intensity of R18 in liposomes alone (dark blue), OMV-Hybrids mixture subjected to five freeze thaw (FT) cycles, or ten FT-cycles (light blue and purple, respectively). **(B)** A non-significant increase in fluorescence intensity was observed after 5 FT-cycles (n = 5). However, a significant increase was observed after 10 FT-cycles (n = 5, p = 0.0454).

intensity for liposomes was measured at 279,112 a.u., while the non-treated negative control displayed a mean intensity of 250,894 a.u. **(Figure S3).** This further underscores the essential role of the freeze thaw method in facilitating lipid mixing and OM-Hybrid formation.

Size analysis of OM-Hybrids: To assess the size of the OM-Hybrids, we employed DLS analysis. Each individual component of the hybrid, namely the OMV and the POPC/PEG-PE liposomes, underwent a freeze-thaw process to ensure accurate evaluation of the treatment's effects. Notably, we observed that the diameter of the OM-Hybrid was 102.3 ± 1.596 nm (mean ± s.d.), while the POPC/PEG-PE liposomes were 70.17 ± 6.907 nm and the OMVs were 140.8 ± 6.675 nm **(Figure 3)**. The average diameter of the OM-Hybrids is in between the mean diameters of each component, indicating that OM-Hybrids are a

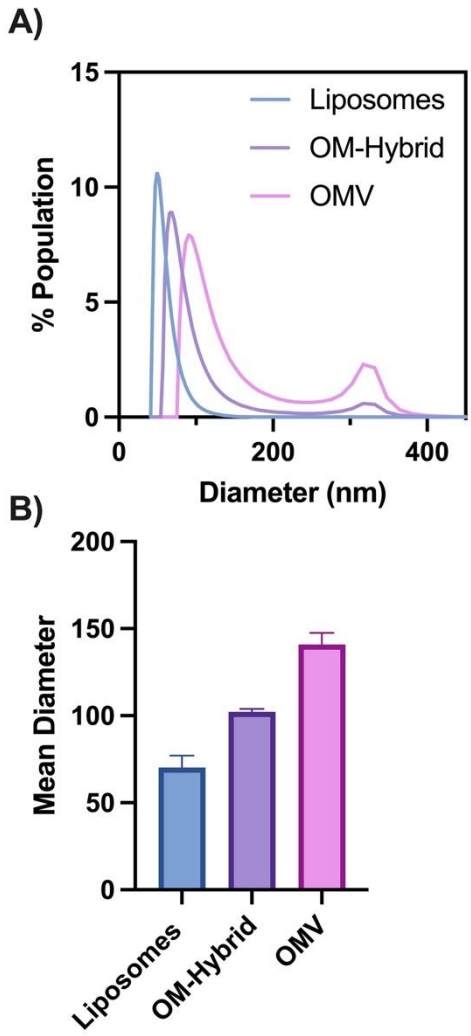

Figure 3: Size of OM-Hybrids and their individual components: (A) Representative diameter distributions of liposomes, OM-Hybrids, and OMVs (shown in blue, purple, and pink, respectively). **(B)** Quantification of the mean diameter of OM-Hybrids and their individual components (n=3).

mixture of these two components. This reinforces the notion that OM-hybrids exhibit properties akin to POPC/PEG-PE liposomes while retaining characteristics of OMV, confirming the occurrence of hybrid fusion.

OM-SB FRAP and diffusion analysis: A key feature of OM-Hybrids is their ability to spontaneously rupture on glass, forming an outer membrane supported bilayer (OM-SB). Membrane fluidity stands as a crucial attribute in biological systems, facilitating pivotal processes such as protein-lipid interactions, molecular transport, and the maintenance of cell membrane integrity. To verify the fluid nature of the OM-SB, our goal was to compare the diffusion coefficient between OM-SB and POPC/PEG-PE SLB by analyzing their respective FRAP profile. OM-Hybrids were prepared with OMVs labeled with DiI, along with unlabeled POPC/PEG-PE, as described in the above section. To ensure a fair comparison of diffusion coefficients between OM-SB and POPC/PEG-PE SLB, we labeled the POPC/PEG-PE liposomes and subjected them to freeze-thaw cycles at a consistent concentration of 0.2 mg/mL, mirroring the lipid concentration found in the OM-Hybrids. OM-Hybrids and POPC/PEG-PE liposomes were ruptured on clean glass surfaces, and the lateral diffusion of their lipids were examined using FRAP **(Figure 4A-B)**. We observed that the OM-SB exhibited a slower fluorescence recovery when compared to the POPC/PEG-PE SLB indicating that diffusion of components in the OM-SB is slower. Furthermore, it was noted that OM-SB exhibited a 76% recovery, while the POPC/PEG-PE SLB showed an 86% recovery of **(Figure 4C)**.

To conduct a more in-depth analysis of the diffusion coefficients for OM-SB and POPC/PEG-PE SLB, we fit the fluorescence recovery data to the single exponential equation, where F_o is the f(t) value (fluorescence intensity) when t (time) is zero, plateau is the maximum recovery, and K is the rate constant which is expressed 1/s[51,52]. Using this fitted equation, we can solve for half-time for recovery ($t_{1/2}$) where $t_{1/2} = \frac{\ln(2)}{K}$.

$$f(t) = F_o + (plateau - Y_o) * \left(1 - e^{(-K*t)}\right)$$

By obtaining the half-time recovery values from the fitted FRAP curves, we were able to calculate the diffusion coefficients for these components using the equation below[53].

$$D = \frac{0.224\ (r^2)}{t_{1/2}}$$

We observed that POPC/PEG-PE SLB had a diffusion coefficient of 0.2619 ± 0.0871 $\mu m^2/s$ while OM-SB had a diffusion coefficient of 0.1242 ± 0.0656 $\mu m^2/s$ **(Figure 4D)**. The lower diffusion coefficient of the OM-SB suggests the presence of biological materials such as proteins and LPS within the bilayer that likely contribute to the reduction in diffusivity of the fluorescent lipids. It is worth noting that these values align with previously reported literature findings. Previously, it was reported that R18 fluorophore in POPC/PEG-PE had a diffusion coefficient of 0.28 $\mu m^2/s$, while the R18 fluorophore in DOPC/PEG-PE had a diffusivity of 0.5 $\mu m^2/s$ [25,26]. Studies by Daniel and coworkers on other planar bacterial membrane formation methods, have reported that the diffusivity of the bacterial outer membrane surface ranged from 0.33 to 0.4 $\mu m^2/s$ depending on the bacterial source of OMVs that were used in the study [25,26]. While these values are higher than those we have observed here, a likely reason for the discrepancy is the use of less of the synthetic lipid component in our method. Since our method uses only 0.2 mg/mL synthetic lipid, hence we see a lower diffusion, indicating a larger fraction of the membrane is of bacterial origin.

We conducted a series of experiments to explore the effectiveness of the freeze-thaw method alone, without synthetic components, in forming a bilayer and causing OMV rupture **(Figure S4).** After subjecting a comparable concentration of OMVs to freeze-thaw-treatment and subsequently adding them to a glass surface, our results revealed that freeze thaw OMVs exhibited adsorption but did not rupture on the glass surface. This underscores the necessity of synthetic lipid integration for bilayer formation. Next, we aimed to determine the minimum concentration of POPC/PEG-PE required to create OM-Hybrids that would rupture effectively. To minimize the use of POPC/PEG-PE liposomes, we tested various concentrations, mixing them with OMVs in equal volumes while varying the liposome concentration **(Figure S5).** Notably, we observed that 0.1 mg/mL did not result in a bilayer formation, producing only adsorbed vesicles. While both 0.5 mg/mL and 1.0 mg/mL liposomes produced bilayers, we found that 0.2 mg/mL was the lowest concentration that allowed us to maintain a substantial amount of the bacterial outer membrane component **(Figure S5)**. Interestingly, we also observed a reduction in bilayer fluorescence intensity as a function of increasing POPC/PEG-PE vesicle concentration **(Figure S5 E-H)**. In our bilayer formation experimental set-up, the fluorophore is exclusively present in the OMV, while the liposomes lack any fluorescent lipid. Consequently, the sole fluorescence source is from the OMVs. As the synthetic lipid concentration

increases during bilayer formation, the fluorescence decreases, signifying a dilution of the OMV component.

Furthermore, assessed the necessity of the freeze-thaw (FT) process for bilayer formation by conducting a negative control experiment in which OMVs and POPC/PEG-PE liposomes were mixed in equal volumes but were not subjected to FT treatment. In this case, we did not observe the formation of a

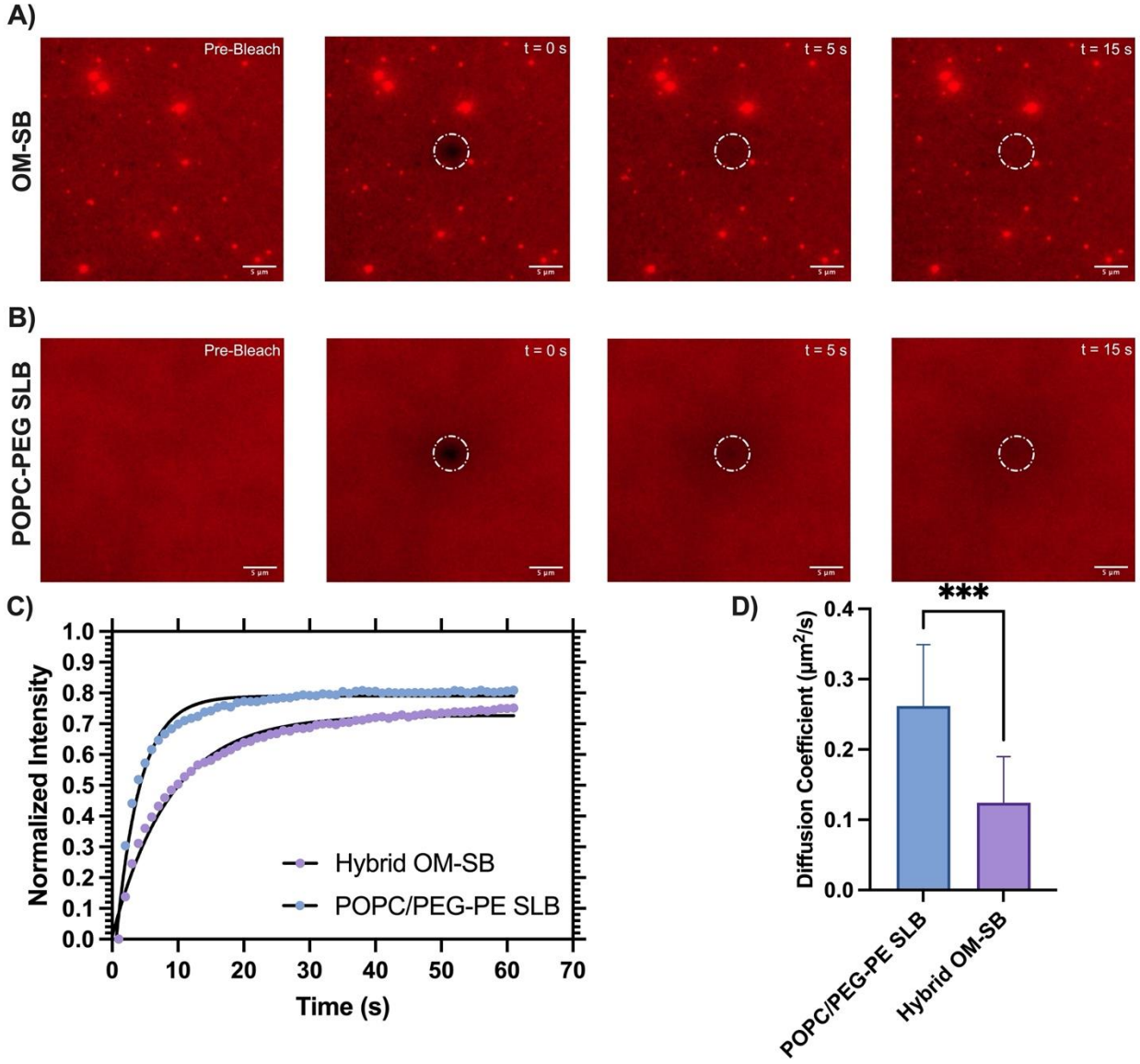

Figure 4: FRAP of OM-SB and POPC/PEG-PE SLB: (A) Fluorescence images of OM-SB **(B)** POPC/PEG-PE SLB during the bleach and recovery stages. The white dashed circle represents the bleach spot. Scale bar in **A** and **B** = 5 µm **(C)** Average FRAP recovery curves of OM-SB and POPC/PEG-PE SLB (n =12). The black lines represent the exponential fit used for analyzing the FRAP curves. **(D)** Diffusion coefficient of the OM-SB and POPC/PEG-PE SLB. A significant decrease in diffusion of OM-SB was observed ($p = 0.0002$; n=12).

bilayer, confirming that the FT treatment is indeed essential for successful hybrid formation and bilayer development **(Figure S6).**

QCM-D analysis of OM-SB formation: To examine the formation pathway and biophysical properties of the OM-SB and POPC/PEG-PE SLB, we used QCM-D with sensor chips coated with SiO_2. QCM-D is not only capable of measuring film thickness but also provides valuable information about the viscoelastic properties of the surface [12]. This dual capability makes it a versatile tool in surface analysis. In QCM-D measurements, a negative frequency shift is indicative of mass adsorption. Additionally, by monitoring dissipation, we gain insights into surface viscoelasticity. For instance, when liposomes or OM-Hybrids first adsorb intact to the surface, an increase in dissipation occurs because these structures can dissipate the vibrational energy of the quartz crystal sensor. However, as these vesicles rupture, a decrease in dissipation is observed, reflecting the planar bilayer's rigidity and inability to dissipate as much energy.

Initially, we conducted an evaluation of the frequency and dissipation characteristics of OMVs adsorbing on SiO_2-coated QCM-D sensors. We observed a final frequency shift of approximately -2.51 Hz and a dissipation of 1.33×10^{-6} **(Figure S7)**. It is noteworthy that OMVs containing LPS tend to exhibit lower frequencies, compared to liposome adsorption, when interacting with the QCM sensor, as LPS can interfere with mass detection, as previously observed with OMVs of *P. aeruginosa* and *E. cloacae*[26,54]. Next, we sought to study the rupture kinetics of POPC/PEG-PE vesicles and OM-Hybrids. We noticed that POPC/PEG-PE liposomes and OM-Hybrids had distinct rupture curves indicating there are differences in the properties of the planar membranes formed from them **(Figure 5)**. Both types of vesicles displayed a comparable two-step rupture process, initially accumulating and then rupturing. However, the final frequencies were different, with OM-SB at -39.5 ± 0.737 Hz and POPC/PEG-PE SLB at -30.3 ± 2.39 Hz **(Figure 5A)**. OM-SB had a larger frequency change, compared to POPC/PEG-PE likely due to the presence of additional components (LPS, proteins, DNA, etc.) associated with the outer membrane of bacteria. Furthermore, our observations indicate that OM-SB exhibits higher viscoelasticity, as evidenced by a dissipation value of $(3.7 \pm 0.098) \times 10^{-6}$, in contrast to POPC/PEG-PE SLB, which has a value of $(1.8 \pm 0.79) \times 10^{-6}$, **(Figure 5B)**. A typical POPC bilayer, without the addition of PEG-PE has a reported dissipation around 0.55×10^{-6} and a frequency shift of around -28.3 Hz[55,56]. While the frequency characteristics of the POPC/PEG-PE SLB align with those of a POPC bilayer, we observe a slight increase in dissipation with the POPC/PEG-PE SLB. We hypothesize that incorporating PEG-PE results water association with PEG, leading to an increase in dissipation.

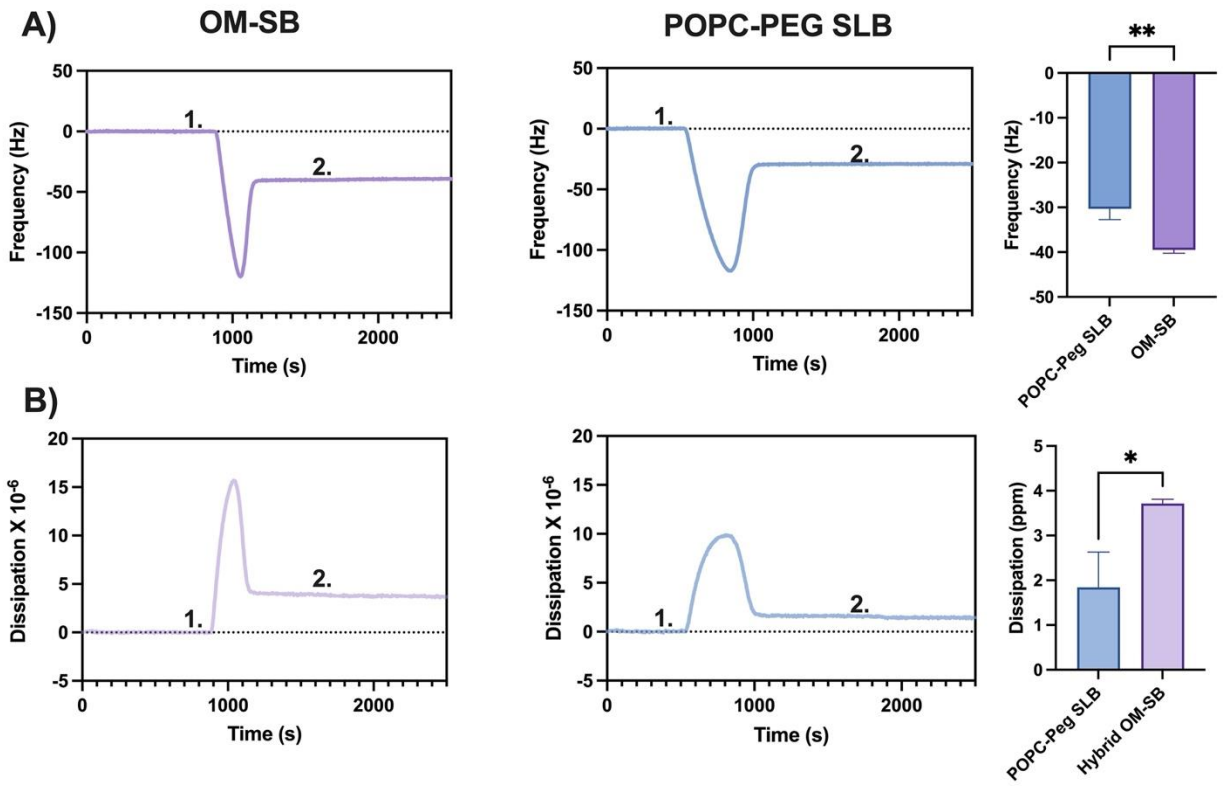

Figure 5: QCM-D of OM-SB and POPC/PEG-PE SLB: (A) Frequency analysis of OM-SB (shown in purple) and POPC/PEG-PE SLB (in blue). Notably, the mean frequency of OM-SB was significantly more negative compared to POPC/PEG-PE SLB (n=3; p=0.0032). (**B)** Dissipation analysis of OM-SB (depicted in purple) and POPC/PEG-PE SLB (in blue). The mean dissipation of OM-SB was higher than that of POPC/PEG-PE SLB (n=3; p=0.0151). In both figures, 1. represents the injection of vesicles (OM-Hybrid or POPC/PEG-PE) into the flow cell, while 2. represents the injection of the wash buffer.

OMV-associated DNA is present on OM-SB: Our objective was to develop a surface that mimics bacterial outer membrane. Consequently, we conducted tests to detect the presence of bacterial OMV components within the surface. Our primary goal was to detect the presence of surface-associated DNA, which is present on OMVs[47,57,58]. To accomplish this, we first created POPC/PEG-PE SLBs and OM-SBs labeled with the DiI membrane dye. Subsequently, we introduced YO-PRO-1, a cell-impermeable DNA-binding dye that fluoresces when bound to nucleic acids. YO-PRO-1 is frequently employed to monitor cell death, as it detects the exposure of cellular DNA during apoptosis[59,60].

Our observations revealed that the dye exhibited binding affinity exclusively to OM-SB, while it did not interact with the POPC/PEG-PE SLB surface. This result establishes that OM-SB contains components characteristic of OMVs **(Figure 6)**. Furthermore, this outcome underscores the gentle nature of our freeze-thaw method, as the DNA associated with bacterial OMVs remained firmly attached to the surface throughout our experimentation. Moreover, this finding provides evidence for the exposure of the

outer leaflet of the bacterial membrane since the dye is membrane impermeable therefore it cannot detect cytoplasmic DNA. This observation aligns with reports suggesting that DNA is situated on the exterior of these vesicles and is reinforced by the impermeability of YO-PRO-1 to the membrane[47,57,58].

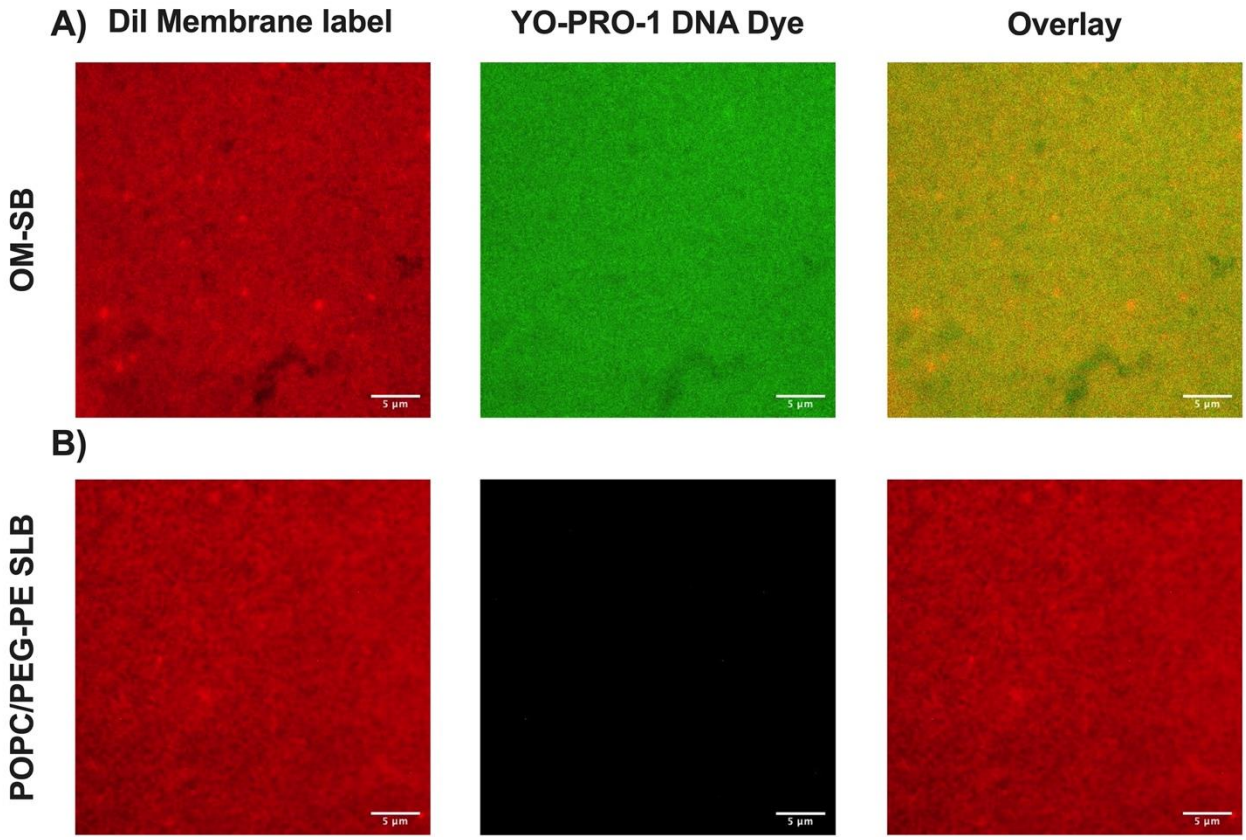

Figure 6: Detection of surface-associated DNA. (A) Fluorescence images of OM-SB. (B) Fluorescence images of POPC/PEG-PE SLB. In both cases, the left column represents the bilayers labeled with DiI membrane stain, the middle column depicts the YO-PRO-1 dye, and the right column is the overlay, indicating specific binding of the YO-PRO-1 to OM-SB. Scale bar in all images = 5 µm.

Conclusion:

Amid the escalating concern surrounding antibiotic-resistant bacteria, there is an urgent demand for expediting drug development processes to effectively address this critical challenge. In this context, we present an innovative approach involving the creation of bacterial OM-Hybrid vesicles which spontaneously rupture on surfaces form a planar bacterial membrane that is compatible with surface-based biosensors, such as QCM-D. Our study reveals a significant breakthrough showcasing that the addition of a minimal amount of synthetic POPC/PEG-PE lipid, just 0.2 mg/mL, is sufficient for OM-Hybrid formation after ten freeze-thaw cycles. The resulting OM-SB membranes exhibit distinct lipid diffusion, and QCM-D frequency and dissipation properties compared to POPC/PEG-PE SLB controls. These variations strongly indicate the presence of biological materials (beyond phospholipids) in the OM-SBs. Intriguingly,

our method also preserves non-covalently associated molecules, as we successfully detected OMV-specific surface-associated DNA in the OM-SB. This discovery reinforces the effectiveness and reliability of our approach in exploring planar surfaces generated from bacterial OMVs. This platform holds considerable significance in the realm of pharmaceutical and microbiological research. First and foremost, it offers an authentic mimic of the bacterial surface environment, providing an ideal substrate for testing and developing new antibacterial agents.

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