Characterization of Giant Plasma Membrane Vesicles containing G Protein-Coupled Receptors for Anchoring to a Novel Surface

Rachel M. Galaska[1], Daniel E. Oseid[2], Giovanni M. Kelly[3], Julie N.L. Albert[3], Anne S. Robinson[2,3]

[1]Department of Chemical Engineering, University of Dayton
[2]Tulane Brain Institute
[3] Department of Chemical and Biomolecular Engineering, Tulane University

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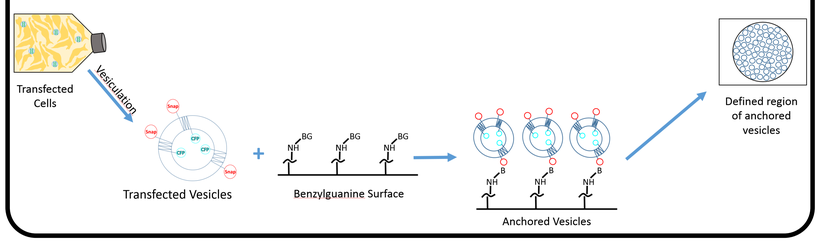
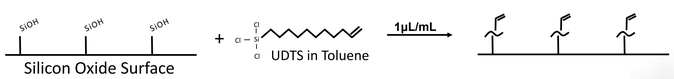
Abstract

Giant plasma membrane vesicles (GPMVs) are micron-sized spheres that retain the membrane composition of the cells they are released from. In this work, we have isolated GPMVs from Chinese hamster ovary (CHO) cells expressing the G-protein coupled receptor (GPCR) adenosine subtype A_{2A}. GPCR-expressing GPMVs were characterized by optical sectioning and three-dimensional reconstruction in a confocal microscope. We also designed a novel brush layer using chlorosilane and thiol-ene chemistry to create a layer of benzylguanine on silicon glass. Benzylguanine reacts rapidly with the mutant 20 kDa DNA repair protein SNAP[1] to form an irreversible covalent bond. Future work is to engineer a functional extracellular SNAP-tag $A_{2A}R$ fusion protein to facilitate GPMV anchoring to our novel surface. Using this system, potentially any membrane protein of interest can be released in GPMVs and anchored for a variety of functions, including biosensing.

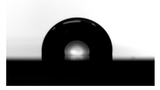


Surface Chemistry

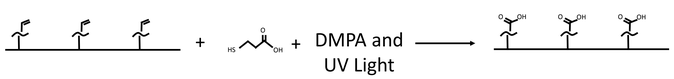
A surface was assembled to anchor the GPMVs. After each additional layer was added, the water contact angle (WCA) was measured to verify the addition was successful, as well as ellipsometry to ensure an increase in brush thickness.



Using chlorosilane chemistry, UDTS in toluene was added. This created a hydrophobic surface.

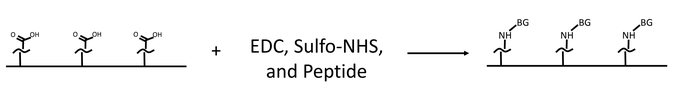


WCA=96°

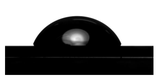


Using thiol-ene chemistry, the surface was then exposed to 3-mercaptopropionic acid, 2,2-dimethoxy-2-phenylacetophenone (DPMA, a photoinitiator), and UV light. This addition caused the surface to become slightly more hydrophilic.

WCA=36.5°



The final step was to add EDC, sulfo-NHS, and benzylguanine. We first added bovine serum albumin (BSA) in phosphate buffered saline in place of benzylguanine. The WCA before the BSA addition was about 10° or less. After BSA addition, the surface became more hydrophobic.



WCA=68.1°

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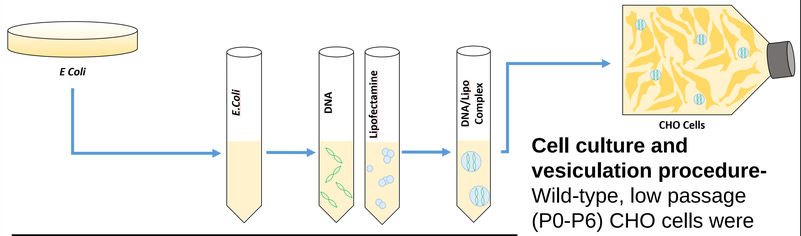
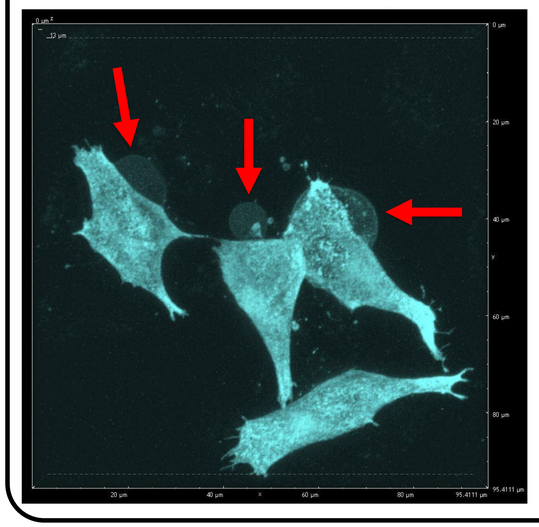
Transfection and Isolation of GPMVs containing $A_{2A}R$-CFP

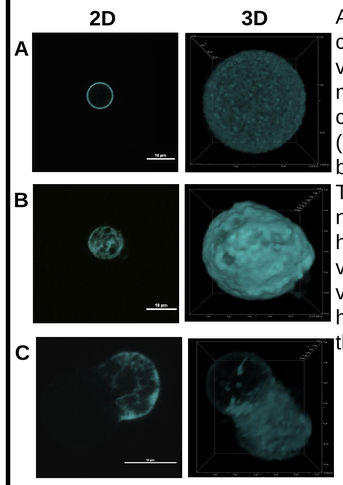
DNA isolation & transfection- A single colony of *E. Coli* (DH5α) containing $A_{2A}R$-CFP in a pCEP4 vector was grown overnight in 5 mls of LB+ ampicillin (50 mg/L) at 37°C and 250 rpm. Plasmid was purified (Promega Miniprep) and freshly isolated pCEP4 (10 µg total DNA/ T25) was combined with Lipofectamine 2000 in Opti-MEM, incubated for 20 minutes at 37°C and added to cells.





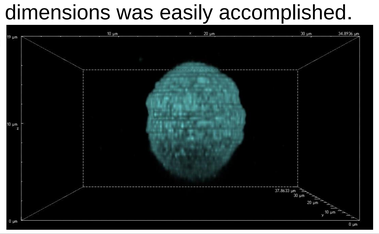
Cell culture and vesiculation procedure- Wild-type, low passage (P0-P6) CHO cells were cultured in DMEM (Corning) supplemented with 10% FBS at 37°C and 5% CO_2. Cells were transiently transfected at 70-80% confluence and grown for an additional 36 hours. After 36 hours of expression, adherent cells were exposed to a buffer[2] containing 25 mM paraformaldehyde (PFA) and 2 mM dithiothreitol (DTT) for one hour at 37°C. Vesicles were collected, protected from light, and stored at 4°C until imaged. **Red arrows** indicate vesicles coming off of CHO cells.

3D reconstruction of GPMVs



A confocal microscope was used to image GPMVs containing $A_{2A}R$-CFP. Three different types of vesicles were observed; Vesicles containing only membrane-bound $A_{2A}R$-CFP (A) ("empty"), vesicles containing $A_{2A}R$-CFP throughout the entire vesicle (B) ("filled"), and a third type that contained both empty and filled vesicles fused together (C). Three dimensional reconstruction of vesicles was necessary to accurately assess diameter without having optical sectioning artifacts. A representative vesicle (D) shows small imperfections due to the vesicle moving during the imaging session, however an accurate estimation of diameter for all three dimensions was easily accomplished.

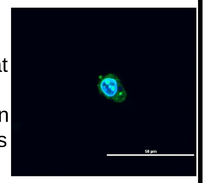
D

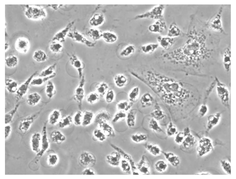


Troubleshooting

Nuclear Dye

Because some isolated vesicles contained $A_{2A}R$-CFP throughout the entire vesicle, we incubated freshly isolated vesicles with a viability reagent (Enzo NUCLEAR-ID Blue/Green[3]) for one hour at room temperature. Interestingly, some vesicles stained positive (blue) for a nuclear dye. This indicates that the GPMV preparation produces some vesicle-like material with intracellular components and this should be taken into consideration depending on the intended application of vesiculation.



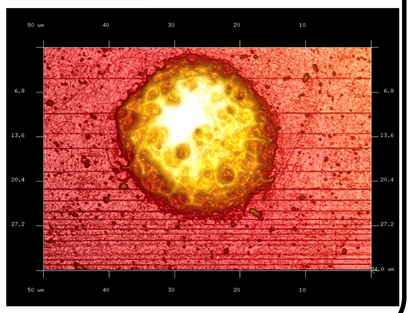


Chloride Salt Vesiculation

An alternative vesiculation process using a hypotonic vesiculation buffer[4] was attempted to eliminate the need for PFA and DTT, which can affect certain downstream applications such as redox chemistry. This procedure, however, took six times longer and produced shrunken cells.

Atomic Force Microscopy

Atomic force microscopy was used to characterize vesicle size and morphology. Concentrated vesicles were placed on a BSA coated glass slide and air-dried for one hour. Vesicle height was much smaller as measured by AFM, most likely due to the dehydration of vesicles after being placed on the slide. Interestingly, we observed distinct holes in the membrane, but this may be an artifact from the AFM tip puncturing the membrane or a confound from the dehydration process.



Conclusions

- **A brush layer was assembled that will later include the addition of benzylguanine to anchor vesicles**
- **G protein-coupled receptors can be isolated in Giant plasma membrane vesicles**
- **Three main types of vesicles were observed based on the localization of $A_{2A}R$-CFP; empty vesicles containing only membrane-bound $A_{2A}R$-CFP, filled vesicles containing $A_{2A}R$-CFP throughout the entire vesicle, and a third type that had both.**

References

1) Keppler A (2003) "A general method for the covalent labeling of fusion proteins with small molecules in vivo." *Nature Biotechnology* 2002 21:86-89
2) Levental (2015) "Isolation of Giant Plasma Membrane Vesicles for Evaluation of Plasma Membrane Structure and Protein Partitioning." *Methods in Molecular Biology* Vol 1232
3) NUCLEAR-ID® Blue/Green cell viability reagent, ENZO Life Sciences, ENZ-53004-C100
4) Del Piccolo N. et al (2012) "Production of Plasma Membrane Vesicles with Chloride Salts and Their Utility as a Cell Membrane Mimetic for Biophysical Characterization of Membrane Protein Interactions." *Analytical Chemistry* 84:8650-8655

Acknowledgements

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