Synthetic Cellular Constructs based on Hierarchical Self-Assembly and Silica Bioreplication

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**Designates undergraduate students/former students and *graduate student
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DTRA, DOE BES, Sandia LDRD, NSF, NIH, NCI Alliance for Nanotechnology in Cancer, AFOSR, Lymphoma and Leukemia Society
Themes and Motivation

• Emulate (proven) biological designs in robust, processable engineering materials – Improve upon Nature by nanostructuring and increased chemical diversity (synthetic ion channels, superhydrophobic surfaces, robust low $k$, synthetic red blood cells)

• Establish general, efficient self- and directly assembly approaches to create, integrate, and understand complex (organic/inorganic) functional materials – Colloidal and Evaporation-Induced Self-Assembly in combination with ALD, layer-by-layer etc.

• Direct assembly of engineered bio-nano interfaces to achieve biocompatibility and achieve tailored material/cellular interactions – Cell-Directed Assembly, Silica Cell Replication, Synthetic Cells, Protocells, etc.
Overall Approach: Combine silica sol-gel chemistry with molecular self-assembly and directed-(top down) assembly to create structurally and functionally hierarchical materials.
Recent work in our group has focused on two classes of synthetic Protocell - Like Objects – Synthesized on Nano or Macro Scales.

**Mesoporous silica nanoparticles**

- Native or synthetic vesicle
- Therapeutic ‘Protocell’
  - JACS 2009, 2009
  - Nature Mater. 2011
  - ACS Nano 2016...

**Silica cell replica**

- Native or synthetic vesicle
- Bio-mimetic ‘Protocell’
  - PNAS 2012
  - Nature Comm. 2014
  - HeLa/HeLa
  - Anti-HCAM (cell adhesion molecule)

**Theme 1:** I will first review our work on the Therapeutic Protocell.
Why NP based drug delivery?

• Nanoparticle-based drug delivery has the potential to package poorly soluble and/or highly toxic drugs, protect them from degradation, and enhance their circulation and biodistribution compared to free drug.

• Furthermore ‘passive’ or ‘active’ targeted delivery promises precise administration of therapeutic cargos to specific/personalized cells or tissues, while sparing collateral damage to healthy cells/tissues and potentially overcoming multiple drug resistance mechanisms.

• Potential to Deliver the undeliverable and re-purpose drugs that failed clinical trials due to toxicity etc.

Therapeutic “Protocell”
Goal: Develop a **Platform Technology** for targeted delivery of drugs to cancer and other diseased cells and tissues – *What are the criteria?*

- Ability to Encapsulate Disparate Cargos
- Sufficient Cargo Capacity
  - Delivery of high concentrations of chemotherapeutic agents to the cytosol of cancer cells can circumvent or overwhelm *multiple drug resistance* (MDR)
- Controllable Release Rates, Endosomal Escape, and Intracellular Targeting of Cargo
- In Vivo Stability and Enhanced Circulation
  - Minimize non-specific uptake and immunogenicity by controlling particle size and modification of the nanocarrier surface
- Biocompatibility/Biodegradability
  - Degradation products must be non-toxic, e.g. Si(OH)$_4$...
- Specificity for Actively Targeted Systems
  - Receptor must be *overexpressed* (10$^4$-10$^5$ copies/cell) on target cells relative to normal cells
    - Targeting Ligands: antibodies, peptides, aptamers, vitamins (e.g. folate) etc
  - Multivalent binding effects can help increase targeting efficacy
  - Receptor should be *internalized* to increase the therapeutic index


Mesoporous Silica NP-Supported Lipid Bilayers (aka ‘Protocells’) simultaneously address the multiple challenges associated with targeted delivery.
APPROACH: PROTOCELL – Combine synergistically the cargo capacity and diversity of mesoporous silica nanoparticles with features developed within liposomes over past 50 years.

Mimicking natural cellular systems we contain, direct, and release cargo employing supported lipid bilayers (SLBs) – stabilized by mesoporous silica nanoparticles.

 APPROACH: **PROTOCELL** – Mesoporous Silica Nanoparticle Supported Lipid Bilayer - *Synergistically combines features of liposomes and mesoporous particles*


**Targeting Peptide (SP94)**
H$_2$N-SFSIIHTPILPLGGC-COOH

**Control Peptide**
H$_2$N-FPWFPLPSPYNGGC-COOH

**Crosslinker**

**Endosomolytic Peptide (H5WYG)**
H$_2$N-GLFHAIAHFIHGGWHGLIHGWYGGGC-COOH

**CARGO**
- Quantum Dot Nanoparticle
- Doxorubicin
- Diphtheria Toxin siRNA
- 5-Fluorouracil
- Cisplatin

*Newly discovered, re-purposed and combined*

DOPC $T_m = -20{^\circ}C$
30% cholesterol, 5%DOPE, 5%PEG
Amorphous mesoporous silica nanoparticle core is synthesized by sol-gel chemistry combined with evaporation induced or colloidal molecular self-assembly – CJB group pioneered silica sol-gel processing in 1980’s.
Key Concept for Self-Assembly: Employ acidic sol-gel conditions that suppress silica condensation and allow high fidelity replication of surfactant mesophases.

Evaporation-Induced Self-Assembly is conducted under dilute aqueous conditions at pH = 2-3 where silicic acid $\text{Si(OH)}_4$ remains monomeric and self-assembles with amphiphilic structure directing agents into liquid crystalline mesophases.

Ralph Iler, The Chemistry of Silica, 1979
EISA: Use evaporation to drive self-assembly of periodic surfactant mesophases under thermodynamic control – water progressively replaced with silicic acid Si(OH)₄ by evaporation.

Micelles act as nanocontainers

Silicic Acid+ other hydrophilic precursors - silicic acid serves as non-volatile hydrophilic fluid

Detergent phase diagram adapted from Scriven and Davis

EISA: Self-Assembly + Sol-Gel Processing + Evaporation → the First Ordered Mesoporous Silica Films and Particles following Kresge et al MCM 41 (Nature ’92)

Membrane

Sensor

low k

Drug delivery

Lu et al., Nature 1997

Ag/Silica

Antimicrobial/Catalyst

Phase Transition

Sea-Shell

Patterns

Sellinger et al., Nature 1998

Fan et al., Unpublished

Doshi et al., Science 2000

Fan et al., Nature 2000
First Generation Mesoporous Silica Cores were synthesized via Aerosol-Assisted Evaporation-Induced Self-Assembly (EISA) – broad PSD

Solvent evaporation driven radially-directed self-assembly

Control residence time and temperature to vary extent of condensation


TEOS, EtOH, H₂O, HCl surfactant (C₀<< CMC)

Extract or calcine surfactants to create mesopores

EISA confined to aerosol yields smooth, spherical or faceted mesoporous silica NPs
Second Generation protocell cores are synthesized by Colloidal self-assembly - allows synthesis of monosized silica nanoparticles (MSNPs) needed for directing BD.

Monodisperse Colloidal stability High surface area (1000 m²/gm)

Aerosol –EISA mesoporous silica cores

Townson, Lin, CJB et al JACS 2013
Second Generation protocell cores are synthesized by Colloidal self-assembly - allows synthesis of monosized silica nanoparticles (MSNPs) needed for directing BD
We synthesize Expanded Pore MSNPs – needed, for example, to accommodate nucleic acid and protein components by a biphasic, oil – water stratification approach.

Scheme 1. Synthesis Process of the 3D-Dendritic MSNSs and Mechanism of Interfacial Growth

- Silica Source: TEOS
- Surfactant: CTAC (cetyltrimethylammonium chloride)
- Swelling agent: Cyclohexane
- Pore size (5-30 nm) controlled by $x = \frac{V_{TEOS}}{V_{cyclohexane}}$
- Particle size controlled by time for each $x$
- We stabilize and conjugate fluorescent labels via co-condensation and hydrothermal synthesis

D. Shen et al., Nano Lett. 2014, 14, 923–932

Achraf Noureddine
Stable monosized MSNPs can be engineered with varying pore sizes, shapes, and cores to accommodate/package multiple cargo types and direct bio-distribution and internalization. Pores may be large enough for individual plasmid components, e.g., Cas9 and gRNA. – Plasmids are associated/complexed with MSNP surfaces (Durfee et al. ACS Nano 2016).
Supported Lipid Bilayer can be ‘tuned’ for stability and fluidity

Crosslinker

\[ \text{NH}_3 \]
\[ \begin{array}{c}
\text{O} - \text{P} = \text{O} + \\
\text{O} - \text{P} = \text{O} \end{array} \]
\[ n = 2, 4, 6, 8, 12, 24 \]

pH 7.4

\[ \text{DOPC} \]
\[ T_m = -20^\circ C \]

or \[ \text{DPPC} \]
\[ T_m = 41^\circ C \]

\[ \text{DOPE} \] (or \[ \text{DPPE} \])

\[ \frac{18:1 \text{PEG-2000 PE}}{} \]

\[ \text{Cholesterol} \]

Model system: HCC Hep 3B

Supported Lipid Bilayer

Nanoporous Silica Core

Targeting Peptide (SP94)
\[ H_2N-\text{SFSIIHTPIPLGGC-COOH} \]

Endosomolytic Peptide (H5WYG)
\[ H_2N-\text{GLFHAIAHFIHGWHGLIHGWAYGGGC-COOH} \]

Cargo:
- Quantum Dot Nanoparticle
- Diphtheria Toxin
- siRNA
- 5-Fluorouracil
- Plasmid DNA
- Cisplatin

Selected against Hep3B by phage display*

Protocell Formation Occurs by Vesicle Adsorption, Deformation, and Rupture – Fusion is governed by van der Waals and Electrostatic Interactions described by DLVO theory
We developed an alternative strategy to load negatively charged nucleic acid and protein cargos in MSNs via cationic vesicle fusion – implications for CRISPR

Mesoporous Silica NP
Controllable:
Size 60-150-nm. Zeta-potential = -30 mV
Pore size 2-20-nm

Plasmid – 3-16kb – Negatively charged
Cationic vesicle > 25% DOTAP

DOTAP

(Zwitterionic helper lipid) DOPE

(membrane stability) Cholesterol

(in vivo stability) DSPE PEG$_{2K}$

(Targeting) DSPE PEG$_{2K}$ NH$_2$

DOTAP
DOTAP/CHOL/DSPE-PEG/DOPE = 47/47/2/4

Plasmids packaged via electrostatically mediated assembly
-Spontaneous dissociation/release upon lipid destabilization at low pH
Protocell Formation Occurs by Vesicle Adsorption, Deformation, and Rupture – Fusion governed by van der Waals and Electrostatic Interactions described by DLVO theory – Zwitterionic SLBs confer exceptional colloidal stability despite lower zeta-potential

Cryo-EM
Zwitterionic DPPC/Cholesterol/PEG Vesicle zeta-potential = -2.9 mV

MSN zeta-potential = -28 mV

Cryo-EM
Targeted Protocell zeta-potential = -3.3 mV

50-nm
At Neutral pH (PBS buffer) Criteria for Formation of Monosized, Non-Aggregated Protocells are Lipid:MSN Surface Area Ratios > 1:1 and Ionic Strength ≥ 20 mM*

Established robust processing window for monosized protocells: rate of vesicle fusion exceeds greatly rate of MSNP aggregation – trickier for MSNP with larger PSD
The Criteria for Formation of Monosized, Non-Aggregated Protocells are Lipid:MSN Surface Area Ratios Exceeding 1:1 and Ionic Strength > 0 mM

- For very large pores, the solid fraction of the silica surface is not sufficient to cause vesicle fusion (DLVO), and/or local curvature arrests fusion, or...
- Would divalent ions, e.g. Mg$^{2+}$ or Ca$^{2+}$, promote fusion?

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hydrodynamic core diameter (nm)</th>
<th>Hydrodynamic protocell diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexagonal (2.8 nm pore)</td>
<td>125.87 ± 1.70</td>
<td>147.53 ± 2.02</td>
</tr>
<tr>
<td>Spherical (2.8 nm pore)</td>
<td>116.07 ± 2.35</td>
<td>141.30 ± 0.75</td>
</tr>
<tr>
<td>Spherical (5 nm pore)</td>
<td>118.33 ± 0.76</td>
<td>145.50 ± 0.62</td>
</tr>
<tr>
<td>Spherical (8 nm pore)</td>
<td>139.23 ± 1.15</td>
<td>184.70 ± 1.06</td>
</tr>
<tr>
<td>Rod-like (2.8 nm pore)</td>
<td>142.93 ± 1.53</td>
<td>172.67 ± 1.72</td>
</tr>
<tr>
<td>Spherical EISA (2.8 nm pore)</td>
<td>700.00 ± 24.68</td>
<td>715.20 ± 49.79</td>
</tr>
</tbody>
</table>
How do we rapidly assess in vivo colloidal stability and targeting specificity of NPs in a relevant model system?

Collaboration with Hon Sing Leong now @ Mayo Clinic
Chick Chorioallantoic Membrane (CAM) Ex ovo Avian Embryo Model serves as accessible model system in which to examine NP stability and targeting in a complex biologically relevant medium.
CAM is composed of vascular networks of diverging and converging flows – it recapitulates features of the flow pattern in organs like liver and spleen.
‘Hydrodynamics’ of monodisperse, stable PEG/TMS MSNPs in arterial network of CAM – *Instant Gratification* for establishing NP structure / property relationships

PEG-trimethylsilyl modified MSN – stability standards
Synthetic Design of Size, Charge, and Colloidal Stability Matched MSNPs

PEG/PEI ‘Patchy’ charge

PEG/N⁺ ‘Uniform’ charge

PEG-silane Mw: 550-750
PEI-silane Mw: 1500-1800
N⁺-silane Mw: 258

: PEI
: PEG

Synthetic condition: PEI/PEG = 1/14

: Quaternary amine

: PEG

Synthetic condition: N⁺/PEG = 1/1.7
Characterization-DLS, Zeta, and Long-Term Colloidal Stability

in D.I. water at 1 mg/mL

Intensity (%)

Hydrodynamic size (nm)

DLS: 60 nm

Normalized counts

Zeta: +40 mV

PEG/PEI

PEG/N^+

Hydrodynamic size (nm)

Aging time (hours)

PEG/PEI

PEG/N^+

D.I.

PBS

DMEM

DMEM+10% FBS

Hydrodynamic size (nm)

Aging time (hours)
In Vivo Binding Difference-PEG/PEI and PEG/N⁺

PEG/PEI (Green) ‘patchy’ charge results in immediate non-specific binding

PEG/N⁺ (Red) ‘uniform’ charge shows stable circulation

PEG/PEI (Green) CAM

PEG/N⁺ (Red) CAM
In Vivo Binding Difference-PEG/PEI and PEG/N\textsuperscript{+}(co-injection)

Co-injection NPs (merged image)
PEG/PEI-Red
PEG/N+- Green

PEG Quaternary amine particles (green) circulate while size and charge matched PEG-PEI (red) non-specifically bind to endothelial cells and are rapidly sequestered by white blood cells.
CAM results verified in vivo in Rat Model using $^{111}$In SPECT Imaging

Single photon emission computed tomography

Dogra, Butler, Brinker et al. *Nature Communications*, 2018
Challenges in leukemia targeting (similar issues exist for treating infectious disease)

- Leukemia is a disseminated disease which requires active targeting to treat circulating cells
- The enhanced permeability and retention (EPR) effect where particles accumulate due to leaky vasculature has limited utility in this disease
- Active targeting demands *in vivo* nanoparticle stability for prolonged circulation and binding to individual cells
- **Combined Properties of effective targeted nanocarrier for leukemia/disseminated disease:**
  - Uniform and controllable particle size and shape
  - High capacity for and precise release of diverse therapeutic cargos
  - High colloidal stability under physiological conditions
  - Minimal non-specific binding interactions
  - High specificity for disease cells
  - Low cytotoxicity
To prove selective targeted binding and delivery, we modify protocells with anti-EGFR antibodies and test their delivery to engineered EGFR+ REH Leukemia Cells in CAM

Scheme 1 – Schematic depicting lipid vesicle fusion onto nanoparticles to form mesoporous silica-supported lipid bilayer nanoparticles (protocells). Drug (gemcitabine) and/or fluorescent molecular cargo (YO-PRO®-1) loaded protocells were assembled by soaking nanoparticle cores with cargo for 24h in aqueous buffer. Liposomes composed of either pre-targeted (DSPC:chol:DSPE-PEG2000-NH2 – 49:49:2 mol ratio) or non-targeted (DSPC:chol:DSPE-PEG2000 – 54:44:2 mol ratio) were then fused to either loaded or unloaded cores. Leukemia cell targeting ability was added to the protocell by successive modifications to the DSPE-PEG2000-NH2 supported lipid bilayer component resulting in highly specific EGFR-targeted protocells. Lipid bilayer and supported lipid bilayer thickness is nearly identical as shown in cryogenic TEM images.

Durfee et al ACS Nano, 2016
We observe selective dose-dependent killing of REH-EGFR cells at 24 hours with no effect on parental REH- cell line – a hallmark of targeted delivery.

Durfee et al., ACS Nano 2016
CAM imaging of loaded ~110-nm Ab-Targeted Protocells 30- minutes post-injection - shows circulation with no apparent non-specific binding or uptake by white blood cells
Using intra vital imaging in the CAM, we can follow the successive steps of targeted Protocell (red) binding to individual circulating leukemia REH-EFGR+ cell targets (green).

EGFR positive REH leukemia cells (green) are rapidly targeted (<5mins) by EGFR-antibody targeted monosized protocells (red). 30 Frames per second. *ACS Nano 2016*
Using intravital imaging in the CAM we visualized protocell binding and intracellular delivery of a cell impermeant drug surrogate (YOPRO).

EGFR positive leukemia cells (blue) show retention of targeted protocells (red) but no delivery of YOPRO cargo (green) at 4hrs. However by 16 hours, targeted cells show intracellular delivery of cargo.

Durfee et al ACS Nano 2016: Current study being conducted at Children’s Hospital of Philadelphia.
Theme 2: Biomimetic Rebuilding of Multifunctional Red Blood Cells: Modular Design Using Functional Components

FNANO Virtual Meeting May 5, 2020

Jimin Guo, PhD UNM
Wei Zhu, Asst. Prof.
South China
University of Technology

Rita Serda, Jacob Ongudi Agola, Achraf Noureddine, Evelyn Ploetz, Stefan Wuttke, Kim Butler
Consider silica sol-gel chemistry in natural systems – *diatoms* have been a fascination since the invention of the microscope, but how do they form? *Peptide catalyzed silica condensation - tested by hydrolysis and condensation of TMOS by proteins extracted from diatoms etc.*
So far...A variety of proteins/enzymes with differing pIs (isoelectric points) have been extracted from diatoms and have been shown to direct silica condensation to produce globular silicates

Table 1  Silica precipitating ability of various enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH</th>
<th>Product</th>
<th>Physical state of solid silica</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>—</td>
<td>Gel</td>
<td>Bimodal nanoparticles 100–200 nm + 700–950 nm</td>
<td>9 hours 10 minutes</td>
</tr>
<tr>
<td>Trypsin</td>
<td>10.5</td>
<td>Solid</td>
<td>Bimodal nanoparticles 100–200 nm + 700–950 nm</td>
<td>9 hours 10 minutes</td>
</tr>
<tr>
<td>Papain</td>
<td>8.8–9.6</td>
<td>Solid</td>
<td>Nanoparticles 500–650 nm</td>
<td>15 minutes</td>
</tr>
<tr>
<td>Bromelain</td>
<td>9.6</td>
<td>Solid</td>
<td>Monolith</td>
<td>25 minutes</td>
</tr>
<tr>
<td><em>Tritirachium album</em></td>
<td>8.9</td>
<td>Solid</td>
<td>Monolith</td>
<td>1 hour</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>7.5</td>
<td>Solid</td>
<td>Monolith</td>
<td>15 minutes</td>
</tr>
<tr>
<td><em>Candida antarctica</em></td>
<td>4.5</td>
<td>Gel</td>
<td>Monolith</td>
<td>9 hours</td>
</tr>
<tr>
<td>Lipase A (CAL)</td>
<td>4.5</td>
<td>Gel</td>
<td>Monolith</td>
<td>8 hours</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>4.5</td>
<td>Gel</td>
<td>Monolith</td>
<td>7 hours</td>
</tr>
<tr>
<td>Rennin</td>
<td>4.5</td>
<td>Gel</td>
<td>Monolith</td>
<td>7 hours</td>
</tr>
<tr>
<td><em>Rhizopus oryzae</em></td>
<td>6.9</td>
<td>Gel</td>
<td>Monolith</td>
<td>7 hours</td>
</tr>
</tbody>
</table>


Monosilicic acid Si(OH)$_4$, which occurs in natural habitats in concentrations between 1 and 100 mM is silica source - Polyamines may catalyze the polycondensation of silanol groups
We hypothesized that protein display on 3D scaffolds would present a crowded organizational motif that locally concentrates soluble silica and catalyzes condensation to enable formation of elaborate patterns mimicking diatoms.

Multiphoton direct writing of 3D protein scaffolds – Bryan Kaehr

Light from a femtosecond titanium: sapphire laser is sent through a confocal scan box to raster the beam
Scaffolded MPL defined 3D protein architectures direct the formation of arbitrary user-defined silica materials

Cellular bio-molecular membrane proteins/components may similarly direct conformal dimensionally stable silica deposition in cell-directed assembly

Hypothesis: the highly crowded cellular microenvironment can serve as a 3D bio-molecular scaffold of catalysts with which to direct conformal, dimensionally stable silica deposition. Proof?

Organelles: (1) nucleolus (2) nucleus (3) ribosome (4) vesicle (5) rough endoplasmic reticulum (ER) (6) Golgi apparatus (7) Cytoskeleton (8) smooth ER (9) mitochondria (10) vacuole (11) cytoplasm (12) lysosome (13) centrioles

Cryo-TEM Tomography - beta cells preserved in situ in pancreatic islet tissue isolated from mice March et al. PNAS 2004; 101: 5565-5570
**Proof: Silica Cell replication (SCR)**

**How does it work?:** At ~pH 3, Monosilicic acid Si(OH)$_4$ does not self-condense. It can interchange with hydrogen-bonded interfacial water at cellular/biomolecular interfaces and be concentrated and catalyzed amphoterically by proximal membrane associated and globular proteins (and perhaps other components) to form silica in self-limiting process (~10-12nm).

Cell replication recapitulates the cytoskeleton and crowded intracellular space of mammalian cells – a scaffold for bottom-up synthetic biology? and coupled enzymatic reactions?
Use exquisite sensitivity of cells to environmental factors to program cell shape, which is faithfully replicated *in silico*.

Blood cells and their varying morphologies induced by osmotic stress are replicated with high fidelity.

- Increasingly abnormal/crenate morphology resulting from increasing levels of osmotic stress – energy consumed/transduced to alter cell shape and protein expression, which are protected and preserved within silica and transformable to other chemistries.
- Cells can be decorated with NPs etc. prior to shape change.

Scale bars = 1µm
Capture mm-nm scale resolution in complete organism – chicken embryo – use brittle fracture to reveal interior structure

Eye

Midbrain

Developing vertebral column

Liver

Embryonic hepatocytes
‘Brittle’ Fracture of the Organism: Allows us to capture with high fidelity selected regions within the interior the organism – here red and white blood cells within the venous system of the chick embryo liver.
What is the ultimate resolution/precision of the replication process?
What is the ultimate resolution/precision of the replication process?

Feature dimension suggests silica deposition thickness limited to < 6-nm; calcination results in cell replica with ~2-nm precision, AFM featureless at 2-nm scale.

SEM analysis of filopodia: mean width of fixed cells (75 nm), cell/silica composites (86 nm), and silica (79 nm) derived from substrate-bound differentiated AsPC-1.
Conventional Microtome preparation shows morphological details nearly indistinguishable from the parent cells.
Silicification (Si-O-Si) proceeds largely with little perturbation of the hydrogen bonded hydroxyl network – and with preservation of protein associated vibrational features – evidence for water replacement hypothesis and self-limitation.
Surface antigens (epidermal growth factor receptors) remain accessible to 50-nm targeted probe during initial stages of silicification.
Surface Antigens (epidermal growth factor receptors) remain accessible and recognizable to 50-nm targeted probe during initial stages of silicification – are occluded at 22 hours, and re-exposed by HF etching silica.
We probed the accessibility and biofunctionality of cell surface and intracellular antigens, receptors, and bio-markers with molecular dyes, ligands, and antibodies with different molecular weights and characteristic dimensions.

<table>
<thead>
<tr>
<th>Fixed Cells</th>
<th>Silicified Cells</th>
<th>Dried Silicified Cells</th>
<th>Desilicified Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomarker</td>
<td>Position in the cell</td>
<td>Approx. Size, M (kDa)</td>
<td>Radius $R_{\text{min}}$ (nm)</td>
</tr>
<tr>
<td>Anti-EGFR</td>
<td>Membrane</td>
<td>150</td>
<td>3.5</td>
</tr>
<tr>
<td>EGF</td>
<td>Membrane</td>
<td>6</td>
<td>1.2</td>
</tr>
<tr>
<td>Lamp-1</td>
<td>Lysosome</td>
<td>120</td>
<td>3.3</td>
</tr>
<tr>
<td>Cox IV</td>
<td>Mitochondrion</td>
<td>17</td>
<td>1.7</td>
</tr>
<tr>
<td>S6 Ribosomal Protein</td>
<td></td>
<td>32</td>
<td>2.1</td>
</tr>
<tr>
<td>Anti-Actin</td>
<td>Cytoskeleton</td>
<td>43</td>
<td>2.3</td>
</tr>
<tr>
<td>Hoechst</td>
<td>Nuclear</td>
<td>0.5</td>
<td>$\sim$0.5</td>
</tr>
<tr>
<td>EGFR targeted NP</td>
<td>Membrane</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

$R_{\text{min}} = 0.066 M^{1/3}$
(for $M$ in Dalton; $R_{\text{min}}$ in nanometer)[2]
Surface and intracellular antigens remains accessible/recognizable during silicification – upon drying only 0.5-nm probe maintains access – ~10-nm etching restores access

Inherent/Intrinsic pore size of replicated silica less than that of the smallest excluded probe (<1.7-nm) – $\text{N}_2$ sorption isotherm of dried cell shows no accessible porosity
The accessibility and biofunctionality of cell surface and intracellular antigens, receptors, and bio-markers were probed with molecular dyes, ligands, and antibodies with different molecular weights and characteristic dimensions.

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<td>Cox IV</td>
<td>Mitochondrion</td>
<td>17</td>
<td>1.7</td>
</tr>
<tr>
<td>S6 Ribosomal Protein</td>
<td>Ribosome</td>
<td>32</td>
<td>2.1</td>
</tr>
<tr>
<td>Anti-Actin</td>
<td>Cytoskeleton</td>
<td>43</td>
<td>2.3</td>
</tr>
<tr>
<td>Hoechst</td>
<td>Nuclear</td>
<td>0.5</td>
<td>~0.5</td>
</tr>
<tr>
<td>EGFR targeted NP</td>
<td>Membrane</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

We observe remarkable fidelity of preservation of dimensions and biomolecular recognition upon silicification, drying, and etching: stable vaccines, cellular decoys...

A549 Cell following Silicification, Drying, and SiO$_2$ Etching
Dehydration seals the system (surface area disappears) and prevents degradation.

**Long Term Preservation of Enzyme Activity**

- **Alkaline phosphatase**
- **Esterase**
- **Acid phosphatase**

Diagram showing cellular components:
- EGF ligand
- Anti-EGFR antibody
- Plasma Membrane
- Anti-COX IV antibody
- Protease
- Alkaline phosphatase
- Acid phosphatase
- Lysosome
- Golgi apparatus
- Ribosome
- Mitochondrion
- Endoplasmic Reticulum
- Nucleus
- DNA

Hoechst dye in the DNA groove
Biomineralized Cancer Cell Vaccines – produced from replicas w/o fixation

• If Si(OH)$_4$ and H$_2$O are equivalent, then hypotonic conditions would promote osmotic flux of Si(OH)$_4$ into cell...
• Alternatively or additionally, does freezing of water concentrate Si(OH)$_4$ in regions near the cell surface promoting diffusion into cell?

Rita Serda, Jimin Guo et al Patent Pending
Biomineralized Cancer Cell Vaccines

Silicified BR5-Akt ovarian cancer cells modified with surface PAMPs

IP Injection of BR5-Akt cells: Preclinical model of serous epithelial ovarian cancer

Prevention of tumor engraftment

IP Injection of BR5-Akt cells

- Prevention of tumor engraftment
- The mimics are internalized by dendritic cells (DC), activate the DC, and enhance antigen processing and presentation of cancer neoantigens to T cells.

Rita Serda UNM Cancer Center

PAMPs = pathogen associated molecular patterns
Key Features of Silica Cell Replication

- **pH 3 silicic acid (100mM) does not self-condense** – pH 3 near the isoelectric point of monosilicic acid
- **Silica deposition occurs uniformly inside and outside the cell and is self-limiting** – 3D scaffolded catalytic surface – complementary to LbL surface sol-gel process (Sandhage)
  - what is diffusivity and condensation mechanism?
  - are membranes in tact and does diffusion occur through Na⁺ channels?

- **From the standpoint of ‘sol-gel processing’ cell-silicified structures are remarkably resistant to drying and calcination**
  - Mechanically completely connected and robust (modulus/density scaling?)
  - Absence of high curvature structures that would result in drying and sintering stress
  - Ultra-thin silica layer allows condensation shrinkage to be accommodated in thickness direction

- **Ultimate nanostructure can be featureless and defect-free ~ 2-nm precision**

- **Preserved biomolecular structure (FTIR) and functionality (enzymatic activity and antigen presentation)**
  - Dried replica can be stored and re-activated with water or etching
  - Enzymatic activity preserved for >1 year in dried samples
  - Can we avoid/reverse fixation?

- **Cancer Vaccine Demonstrated**
**Red blood cells (RBCs)** possess many unique characteristics, including **special shape, flexibility, the ability to carry oxygen**, and **long circulation times** – can we create stable, synthetic, long-circulating analogues?

### Table: Red Blood Cells

<table>
<thead>
<tr>
<th>Illustration</th>
<th>Description*</th>
<th>Number of Cell per mm$^3$ (µl) of Blood</th>
<th>Function</th>
<th>Duration of Development (D) and Life Span (LS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Side view (cut)</td>
<td>Biconcave, anucleate disc; salmon-colored; diameter 7–8 µm</td>
<td>4–6 million</td>
<td>Transport oxygen and carbon dioxide</td>
<td>D: 5–9 days LS: 100–120 days</td>
</tr>
<tr>
<td>Top view</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Hemoglobin* is a protein that binds and transports oxygen in red blood cells. It consists of four subunits: two **α** chains and two **β** chains. The hemoglobin molecule is flexible and able to adapt to the curvature of red blood cells, allowing efficient oxygen exchange during circulation. The **top view** of a red blood cell reveals its characteristic biconcave shape, which contributes to its flexibility and efficient oxygen transport.

---

Use exquisite sensitivity of cells to environmental factors to program cell shape, which is faithfully preserved in Red Blood Cell Bio-replicas.

Blood cells and their varying morphologies induced by osmotic stress are replicated with high fidelity and preserved physical dimensions.

- Increasingly abnormal/crenate morphology resulting from increasing levels of osmotic stress – energy consumed/transduced to alter cell shape and protein expression, which are protected and preserved within silica and transformable to other chemistries.
- Cells can be decorated with NPs etc. prior to shape change.

Scale bars = 1µm
Silica cell replication combined with layer-by-layer assembly and native RBC membrane fusion enables construction of a **multifunctional artificial RBC platform**.
Fabrication of Rebuilt red blood cells (RRBCs)

Jimin Guo et al ACS Nano in press
Buffered Etching Process – Inside-out precision etching of silica – residual silica at polymer/replica interface preserves RBC shape

Si content (%)

BOE content (uL)

3% residual

Jimin Guo et al ACS Nano in press
RRBCs maintain identical biconcave shape and charge as native RBCs

Jimin Guo et al ACS Nano in press
RRBCs deform and reconstitute their shape like native RBCs

Jimin Guo et al ACS Nano in press
**Surface Self-Antigens are preserved RRBCs**

<table>
<thead>
<tr>
<th>Blood type</th>
<th>O−</th>
<th>O⁺</th>
<th>A−</th>
<th>B−</th>
<th>A⁺</th>
<th>B⁺</th>
<th>AB−</th>
<th>AB⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen present</td>
<td>Antigen Rh</td>
<td>Antigen A</td>
<td>Antigen B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody-mediated agglutination</td>
<td>Anti-Rh antibody</td>
<td>Anti-A antibody</td>
<td>Anti-B antibody</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Jimin Guo et al ACS *Nano in press*

Right-side-out-membrane orientation of the rebuilt RBC is important in maintaining the same surface property as the native RBC.
CD47 and ICAM-4 ‘Self-Recognition’ antigens preserved on RRBCs

Jimin Guo et al ACS Nano in press
RRBC is **biocompatible** with native RBCs, endothelial cells, and macrophages.

![Graphs showing hemolysis and viability of different cell types with varying concentrations of RRBC](image)

**HUVEC cells**

**Raw 264.7 cells**

Human Umbilical Vein Endothelium (HUVEC) Cells

Jimin Guo et al ACS Nano in press
RRBCs readily circulate in the chicken embryo vasculature

Only RRBCs with sufficiently low modulus and native RBC membrane-like surface properties can sustain long-term circulation times.

Jimin Guo et al ACS Nano in press
RRBCs readily circulate in the chicken embryo vasculature like native RBCs (black ghosts)

RRBC labeled red
Vessel walls labeled green
RBCs unlabeled

50 μm

Jemin Guo et al ACS Nano in press
Elimination half-life of the RRBC was **41.8 h**

Elimination half-life was calculated as

\[ t_{1/2} = \frac{\ln(2)}{\beta} \]

\[ C(t) = Ae^{-\alpha t} + Be^{-\beta t} \]

**In vivo circulation**

**RBC ghosts derived from mouse model – Why 42h? How do we further increase circulation?**
**RRBCs can be loaded with Hemoglobin**

Chitosan layer incubated with hemoglobin prior to RBC ghost fusion

Jimin Guo et al *ACS Nano in press*
RRBC can be loaded with functional cargos and be magnetically sequestered from fluids.

Magnetic rebuilt RBCs

- MRI contrast agent
  - Porphine Manganese
    - 3.5 μg/million particles

- Anticancer drug
  - Doxorubicin
    - 5.5 μg/million particles

Physiological pH

Acidic condition

Extra Polymer layer

Fe₃O₄

15 nm

Jimin Guo et al ACS Nano in press
RRBCs serve as long circulating detox and sensing agents

A)

Hemolysis (%)

Hlα pre-incubated then RBC added

B)

C)

Jimin Guo et al ACS Nano in press
RRBCs synthesized by **four independent synthetic methodologies:** *silica cell bio-replication*, *layer-by-layer assembly* of biocompatible polymers to translate native RBCs into flexible, loadable RBC-shaped polymer cores, *buffered etching* of the silica to adjust the core mechanical modulus, and *encapsulation* within native RBC derived membrane ghosts to establish in vivo ‘colloidal’ stability and avoid recognition by the immune system.

The RBC mimic particle displays **deformability, nearly zero hemolytic activity, low cytotoxicity, and sustained vascular flow** in the Ex Ovo chick chorioallantoic membrane model. In addition, different *functional cargos* (such as hemoglobin, Mn-TPPS4, DOX, iron oxide nanoparticles, biosensors) could be loaded onto the RRBC to provide oxygen delivery, MRI contrast, anticancer, magnetic properties, and toxin sequestration.

RRBC macroparticles may serve as a new tool to promote our understanding and mediation of complex life processes. They may constitute a **new high capacity multifunctional delivery and detection platforms.**
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• Ultimate nanostructure can be featureless and defect-free ~ 2-nm precision

• Preserved biomolecular structure (FTIR) and functionality? (with caveat of fixation)
  - Does de-silicification reveal silica occluded structure and re-generate biofunctionality? 
  if yes dried structure could be stored and re-activated
  - Can we avoid/reverse fixation?

• Self-Consistent Mechanism?
Acknowledgements: Sandia National Laboratory LDRD Program, NIH, NSF, AFOSR

Jimin Guo, Wei Zhu, Rita Serda, Jacob Ongudi Agola, Achraf Noureddine, Evelyn Ploetz, Stefan Wuttke, Kim Butler