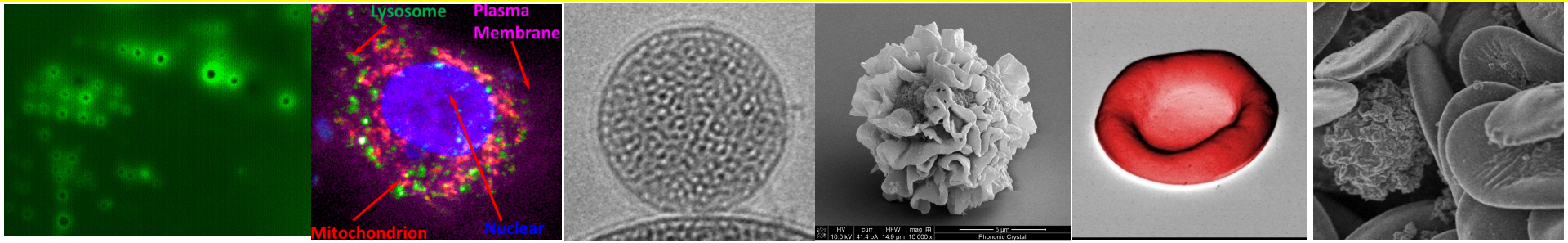
Synthetic Cellular Constructs based on Hierarchical Self-Assembly and Silica Bioreplication

FNANO Virtual Meeting May 5, 2020



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**Designates undergraduate students/former students and *graduate student

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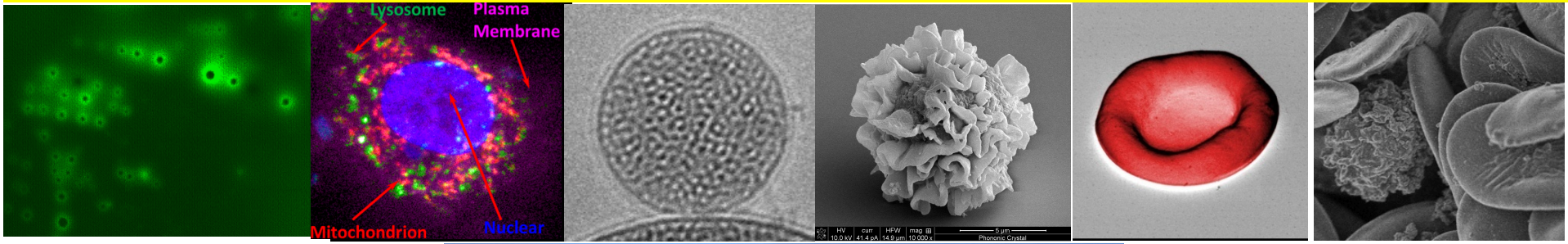
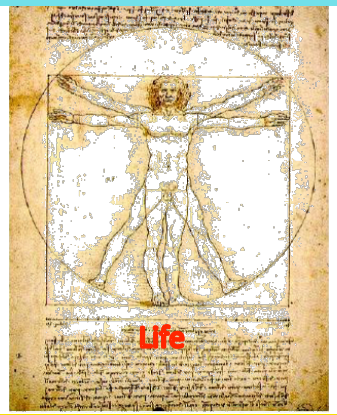
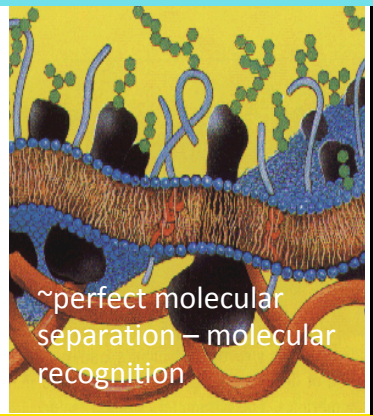


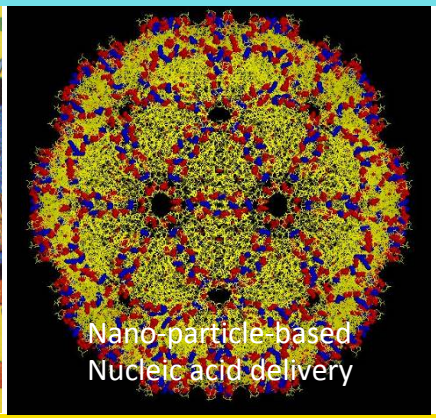
DTRA, DOE BES, Sandia LDRD, NSF, NIH, NCI Alliance for Nanotechnology in Cancer, AFOSR, Lymphoma and Leukemia Society

Biological systems exhibit complex functionality, are composed of nano-scale components, and have evolved to solve challenging engineering problems:

Need robust approaches to assemble disparate materials into hierarchical – length scale prioritized structures







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)

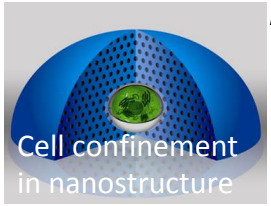
•Establish general, efficient self- and directly assembly approaches to create, integrate, and understand complex (organic/inorganic) functional materials – Colloidal and Evaporation-Induced Self-Assembly in combination with ALD, layer-by-layer etc.

•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.

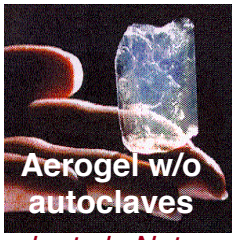
Overall Approach: Combine silica sol-gel chemistry with molecular self-assembly and directed-(top down) assembly to create structurally and functionally hierarchical materials

Sol-gel chemistry - combined with self- and directed assembly

Confinement Induced Quorum Sensing of individual S. aureus
Carnes et al. Nature Chem. Biology 2010

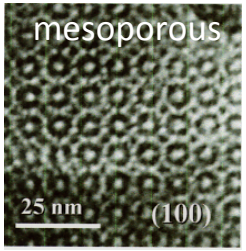


Low thermal conductivity



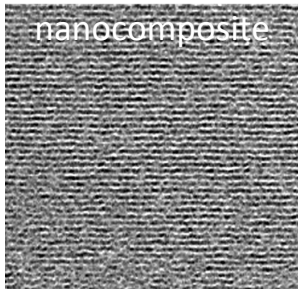
Prakash et al., Nature 1995

Membranes/optimized low k

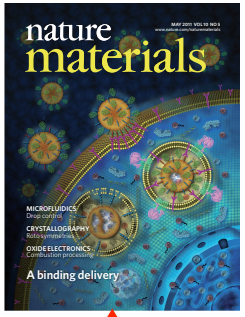


Lu et al., Nature 1997

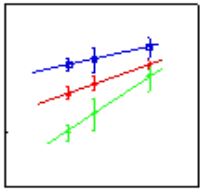
Seashell mimic



Sellinger et al., Nature 1998



Understand nanoconfinement effects on structure and properties



Fan et al., Nature Mater. 2007

Physical/Chemical Isolation Of Individual Cells

Aerogels Via Springback

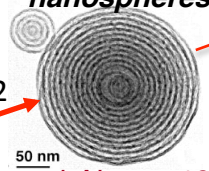
Evaporation Induced self-assembly (EISA)

Multi-component (EISA)

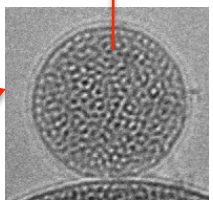
Nano-indentation modeling / spectroscopy

Aerosol or Colloidal Self-Assembly

First fully ordered nanospheres

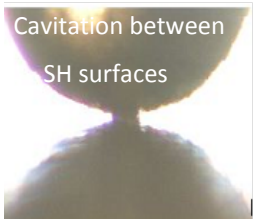


Lu et al, Nature 1999



'Supported Liposomes' Nature Mater. 2011

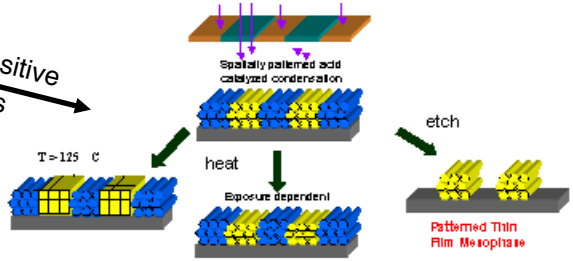
Understand long range hydrophobic forces
Singh et al. Nature 2006



Fractal SH surfaces via springback

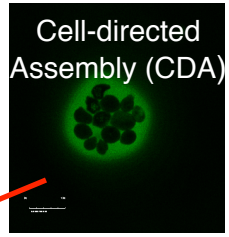
Optical definition of nanoscale structure and function

EISA of photosensitive nanostructures

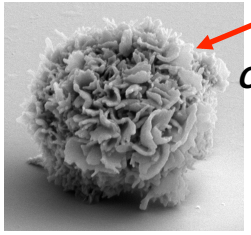


Doshi et al., Science 2000

Novel biotic/ abiotic materials built by cells
Baca et al. Science 2006



Cell-Directed-assembly

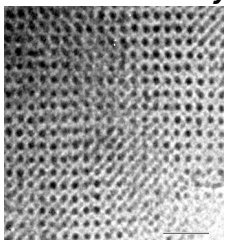


Cell Replicas

PNAS 2012, Nat Comm. 2014

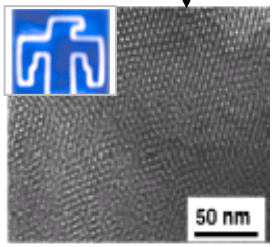
3D nanocrystal self-assembly

Au NP/silica arrays



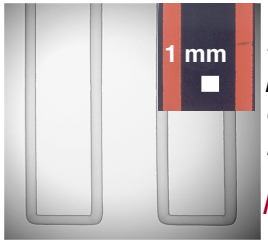
Understand collective electronic, photonic, plasmonic, properties
Fan et al., Science 2004

polymerizable surfactant (EISA) using



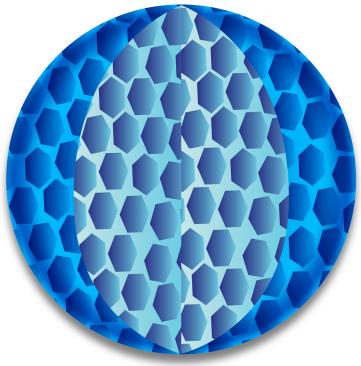
Nanostructured conjugated polymers
Lu et al., Nature 2001

"smart ink"

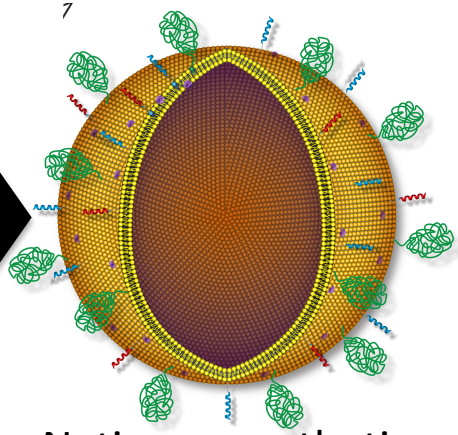


Direct writing of porous & composite nanostructures
Fan et al., Nature 2000

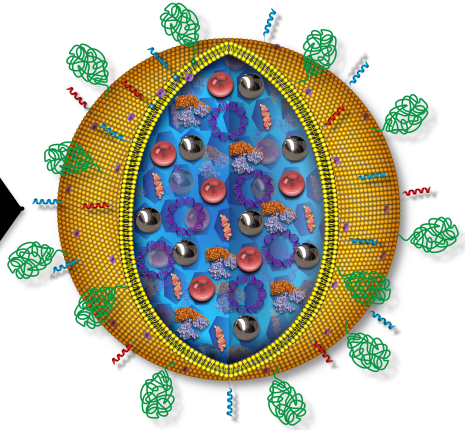
Recent work in our group has focused on two classes of synthetic *Protocell - Like* Objects – Synthesized on Nano or Macro Scales



Mesoporous silica nanoparticles

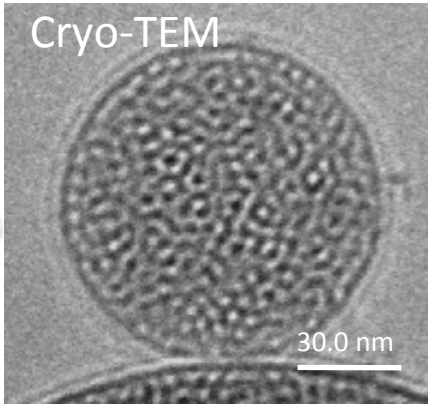


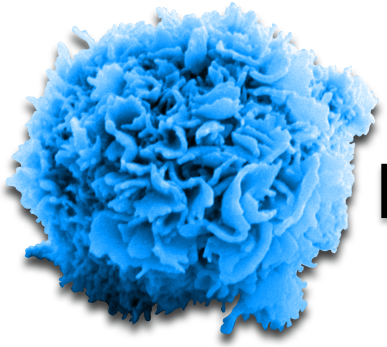
Native or synthetic vesicle



Therapeutic 'Protocell'

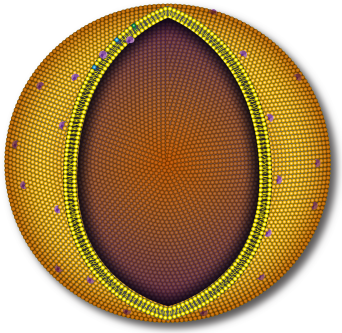
JACS 2009, 2009
Nature Mater. 2011
ACS Nano 2016…



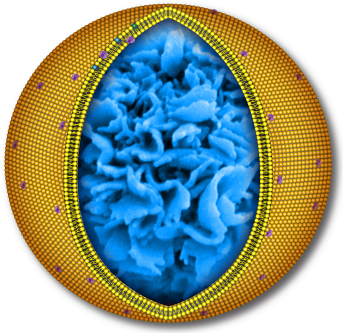


Silica cell replica

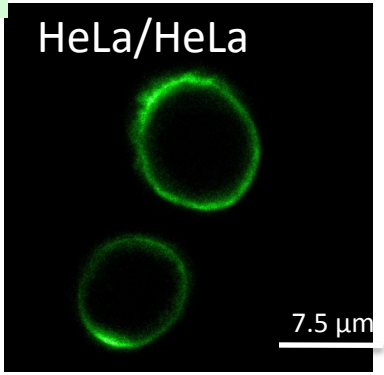
PNAS 2012
Nature Comm. 2014



Native or synthetic vesicle

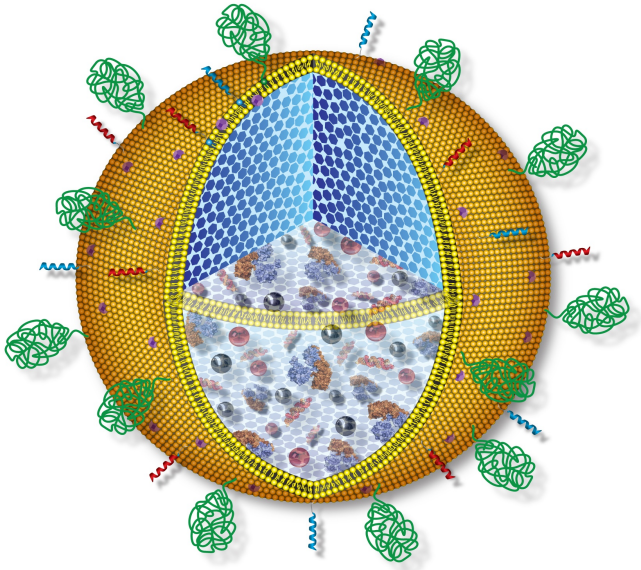


Bio-mimetic 'Protocell'



Anti-HCAM (cell adhesion molecule)

Theme 1: I will first review our work on the Therapeutic Protocell



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.*

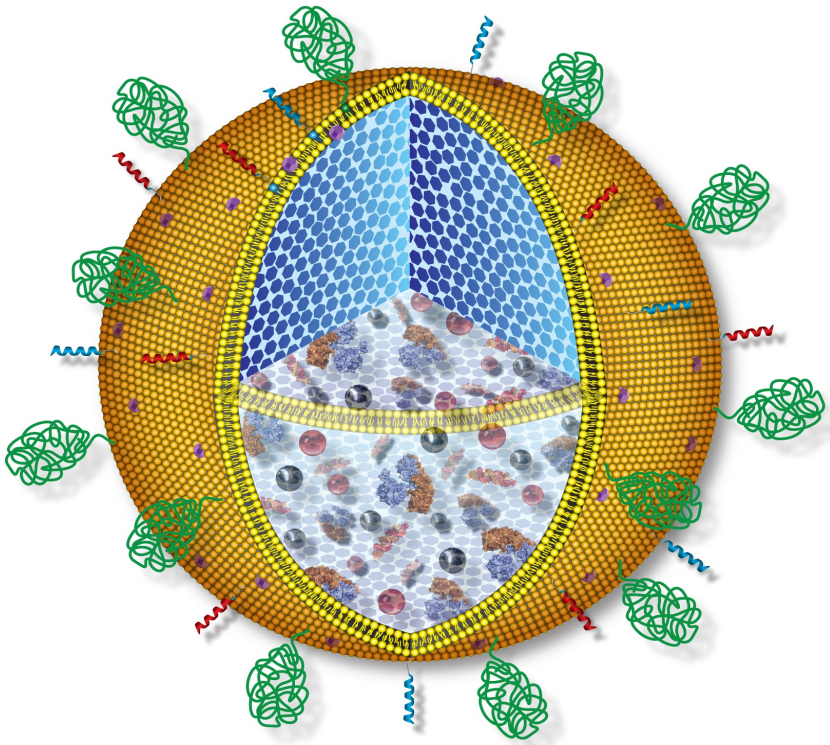
Goal: Develop a Platform Technology for targeted delivery of drugs to cancer and other diseased cells and tissues – *What are the criteria?*

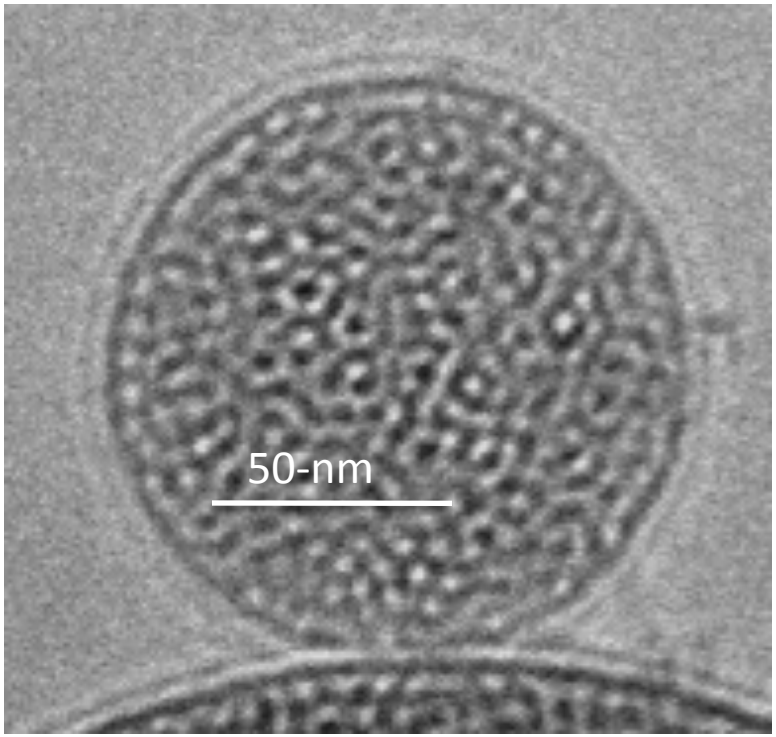
- **Ability to Encapsulate Disparate Cargos**
- **Sufficient Cargo Capacity**
  - Delivery of high concentrations of chemotherapeutic agents to the cytosol of cancer cells can circumvent or overwhelm multiple drug resistance (MDR)
- **Controllable Release Rates, Endosomal Escape, and Intracellular Targeting 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

Nature Nanotechnology (2007). **2:** 751-760

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

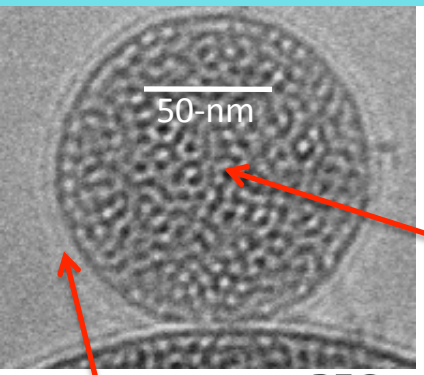




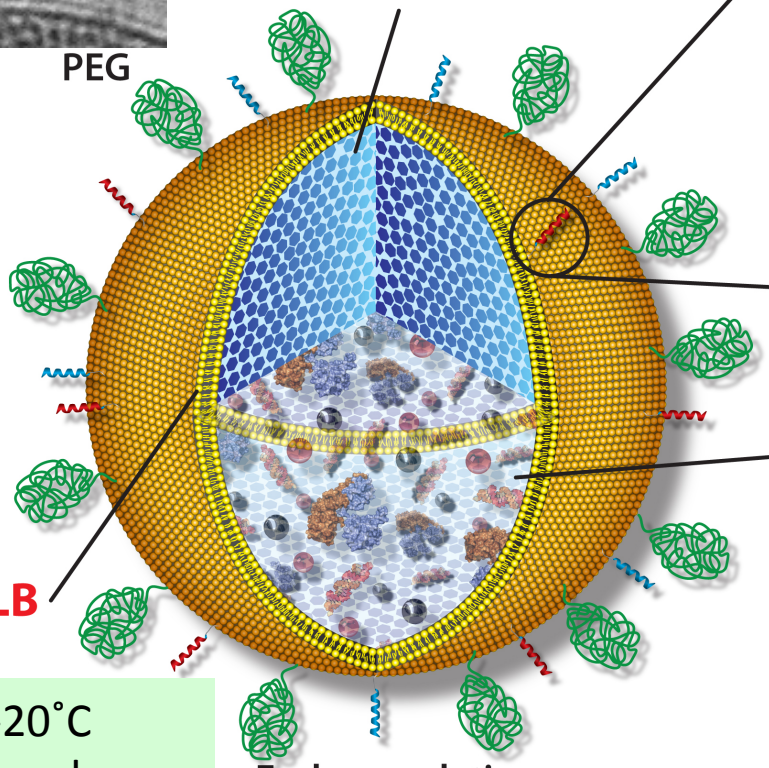
Mimicking natural cellular systems we contain, direct, and release cargo employing supported lipid bilayers (SLBs) – stabilized by mesoporous silica nanoparticles

Liu, CJB *et al.*. *J. Am. Chem. Soc.* 131, 1354-56, (2009); 131, 7567-69 (2009), Ashley, CJB *et al. Nature Mater.* May 2011 (Cover), *ACS Nano* March 2012 (Cover), *ACS Nano* 2016….

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



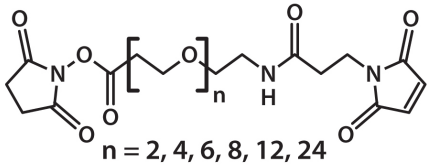
Mesoporous Silica – Evaporation-Induced Self-Assembly, CJB *et al. Nature '97, '99, JACS '09….*Colloidal Self-Assembly, *ACS Nano '16*
(Cryo-TEM – Baylor)



Targeting Peptide (SP94)
H_2N-SFSIIHTPILPLGGC-COOH

Control Peptide
H_2N-FPWFPLPSPYGNGGC-COOH

Crosslinker

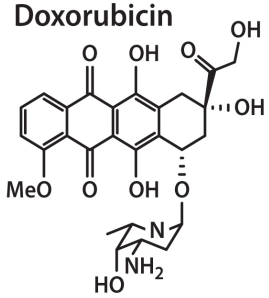


Targeting peptides, anti-bodies, scFv's etc.

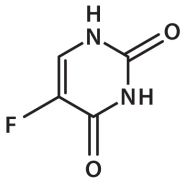
Endosomolytic Peptide (H5WYG)
H_2N-GLFHAIAHFIHGGWHGLIHGWYGGGC-COOH

CARGO

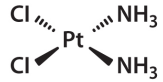
Quantum Dot
Nanoparticle
Doxorubicin



Diphtheria Toxin
siRNA
5-Fluorouracil



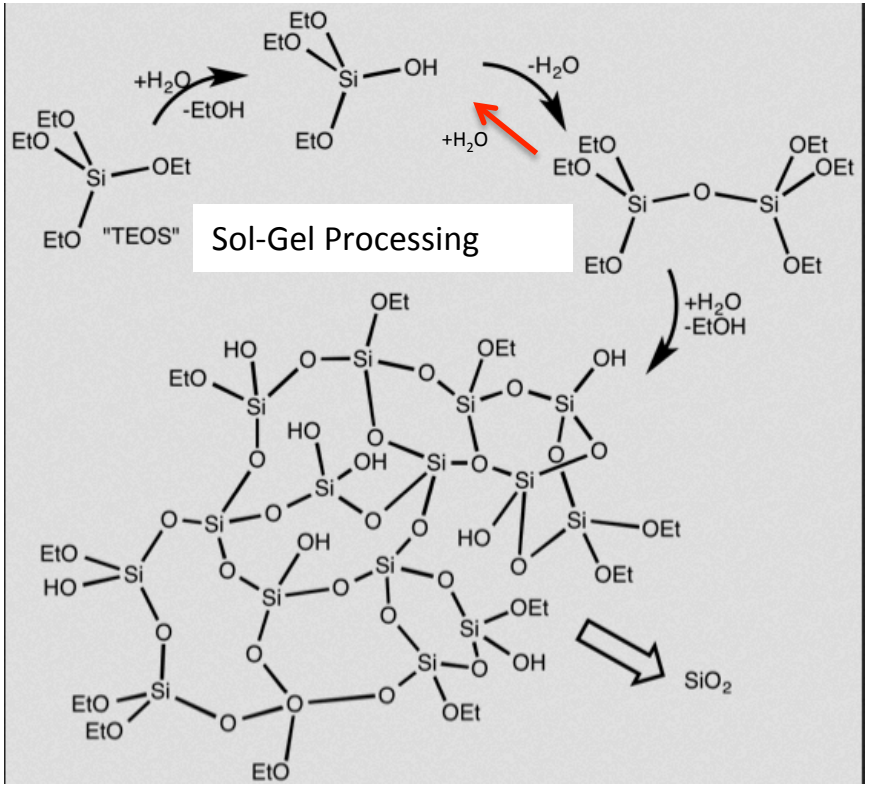
Cisplatin

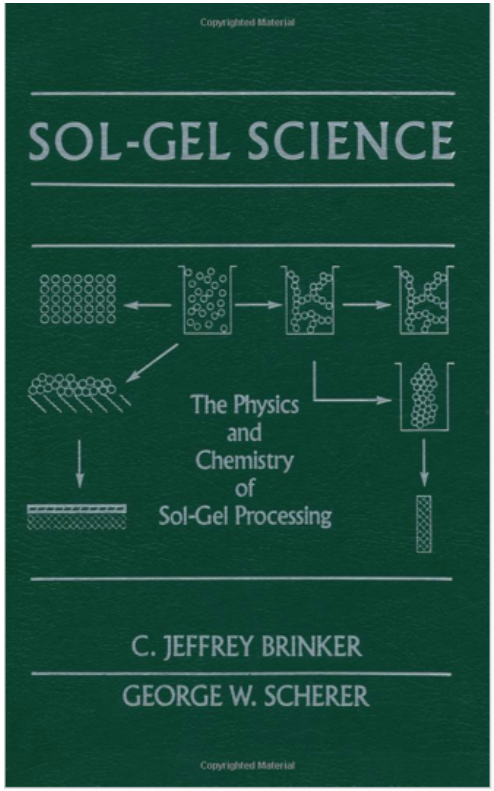


DOPC T_m = -20˚C
30% cholesterol,
5%DOPE, 5%PEG

Newly discovered, re-purposed and combined

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



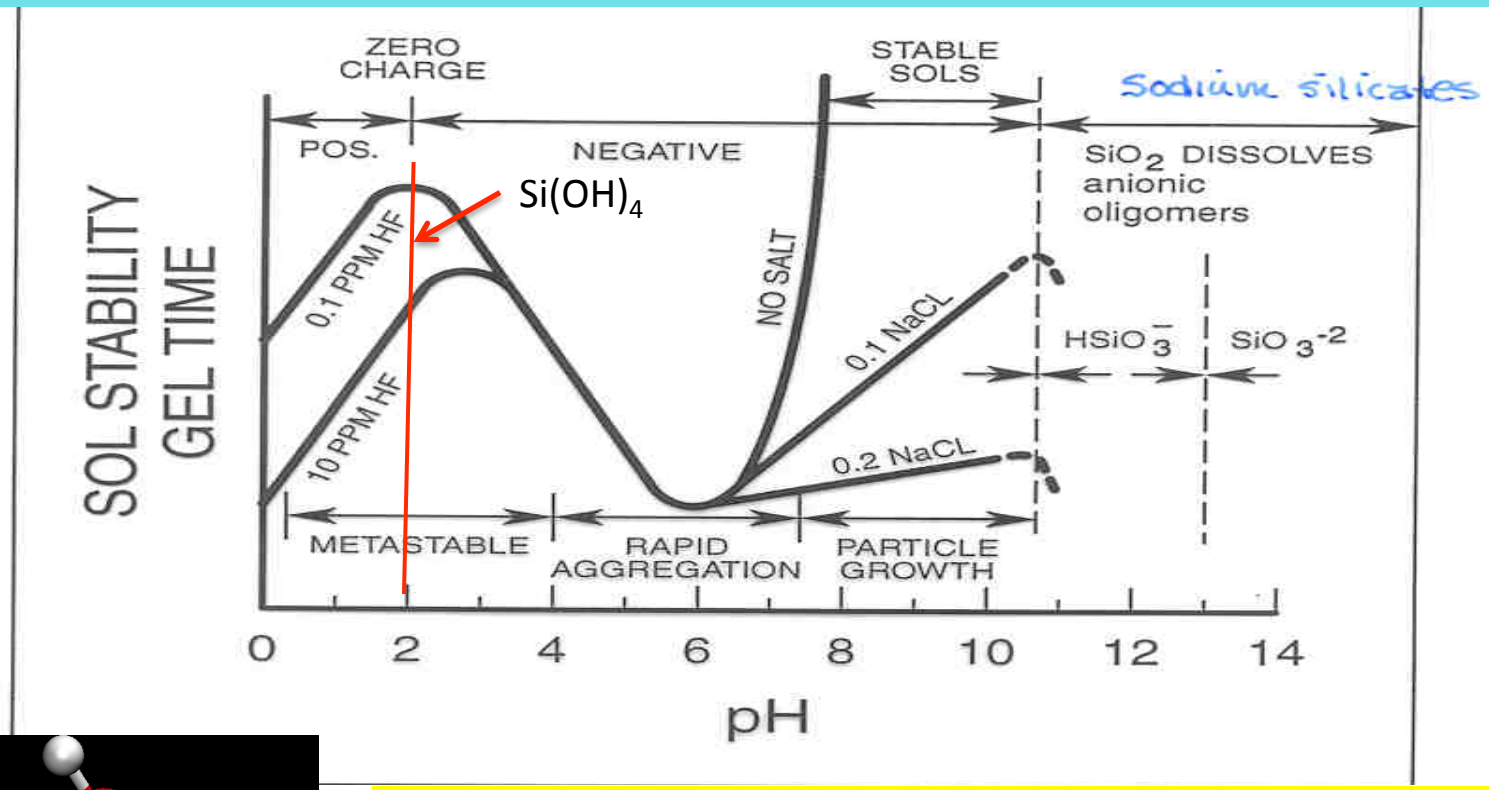


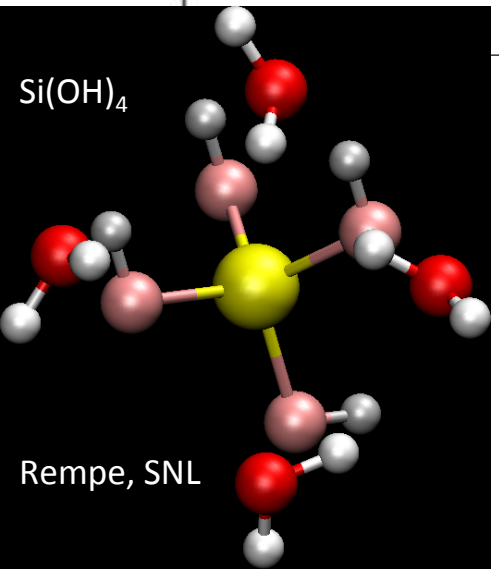
Wikipedia: Sol-Gel Processing: formation of metal oxides via hydrolysis and condensation of soluble molecular precursors – reference 1.

Amorphous silica prepared by sol-gel/colloidal processing is Generally Recognized as Safe (GRAS) by FDA – It degrades to silicic acid $Si(OH)_4$ by hydrolysis – solid silica NPs FDA-approved for imaging (C-dot, Cornell)

1. ^ a b Brinker, C.J.; G.W. Scherer (1990). *Sol-Gel Science: The Physics and Chemistry of Sol-Gel Processing*. Academic Press.

Key Concept for Self-Assembly: Employ acidic sol-gel conditions that suppress silica condensation and allow high fidelity replication of surfactant mesophases

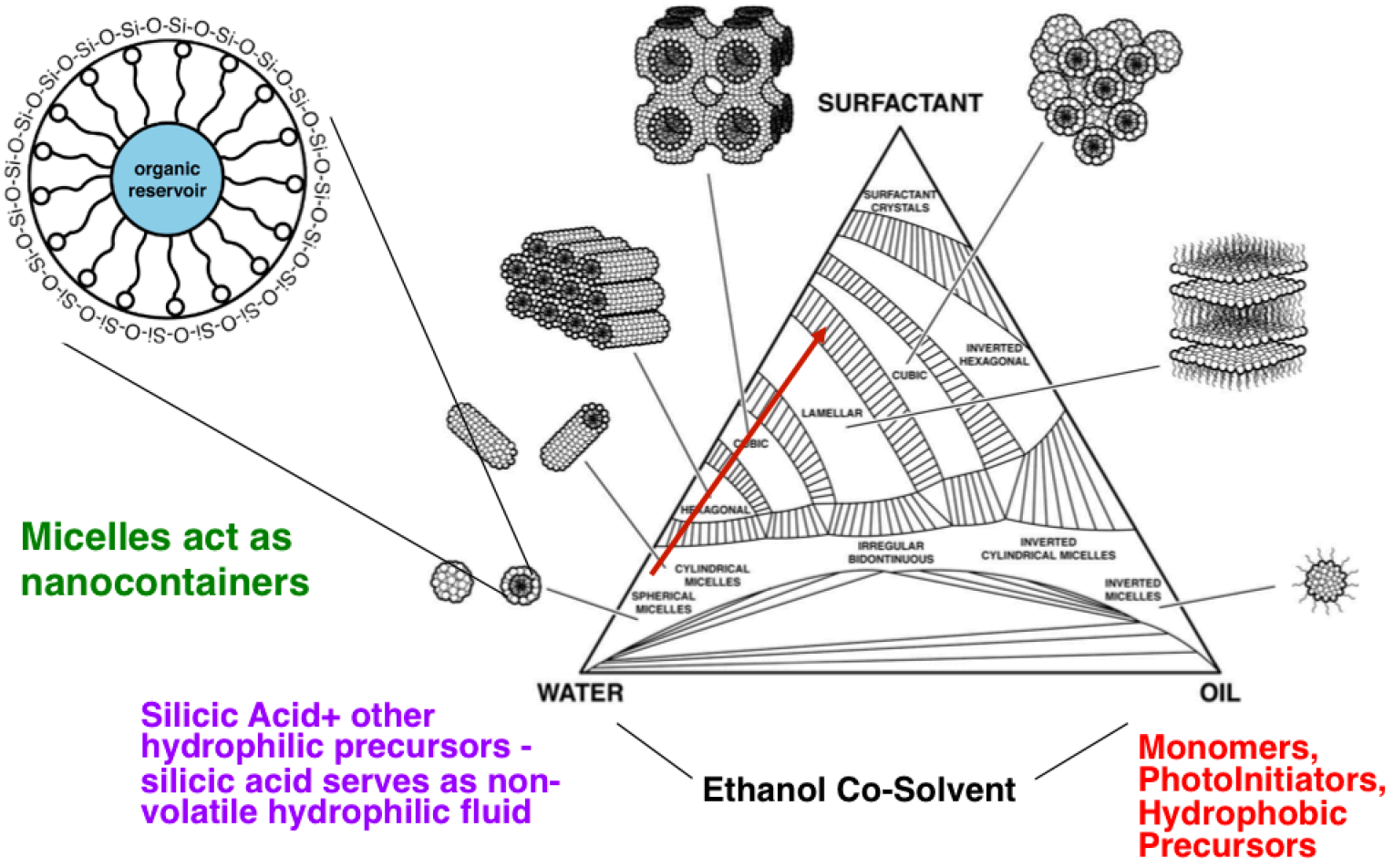




Evaporation-Induced Self-Assembly is conducted under dilute aqueous conditions at pH = 2-3 where silicic acid $Si(OH)_4$ remains monomeric and self-assembles with amphiphilic structure directing agents into liquid crystalline mesophases

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

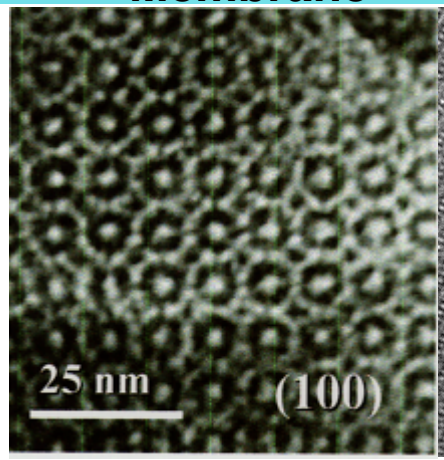


Detergent phase diagram adapted from Scriven and Davis

For further reference, see papers on evaporation induced self-assembly (EISA) of porous (*Lu et al. Nature, 1997*) composite (Sellinger et al. *Nature,* 1998) particulate *(Lu et al.Nature*, 1999) and patterned (*Fan et al. Nature 2000, Doshie et al. Science 2000)* nanostructures

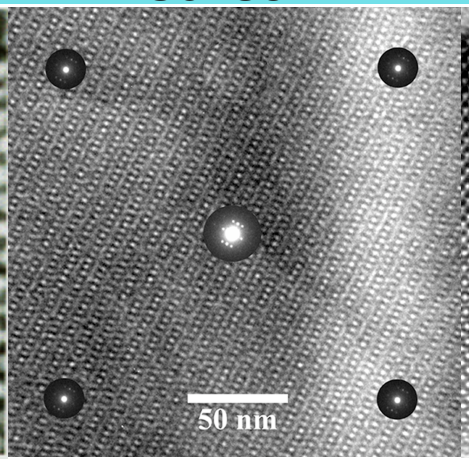
EISA: Self-Assembly + Sol-Gel Processing + Evaporation → the First Ordered Mesoporous Silica Films and Particles following Kresge et al MCM 41 (*Nature '92*)

Membrane

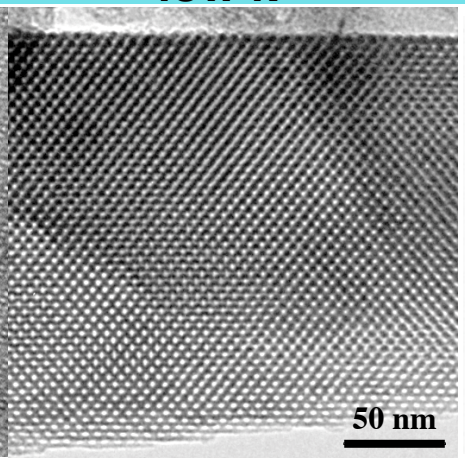


Lu et al., Nature 1997

Sensor

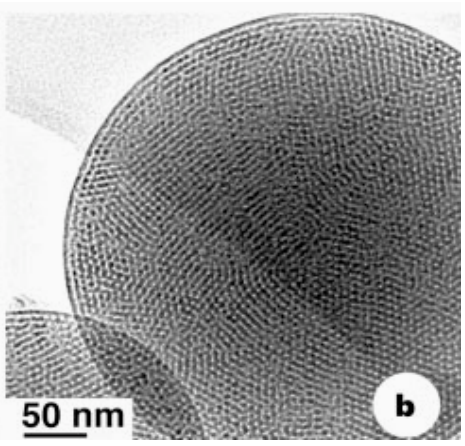


low k



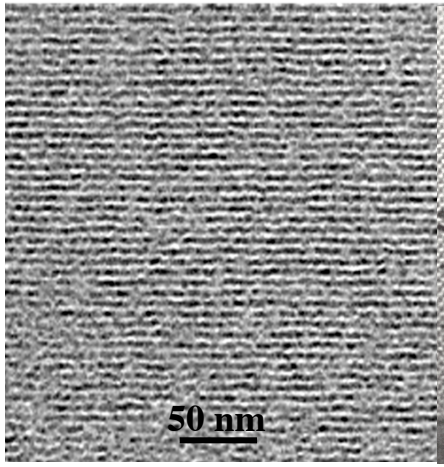
Brinker et al., Adv. Mater. 1999

Drug delivery



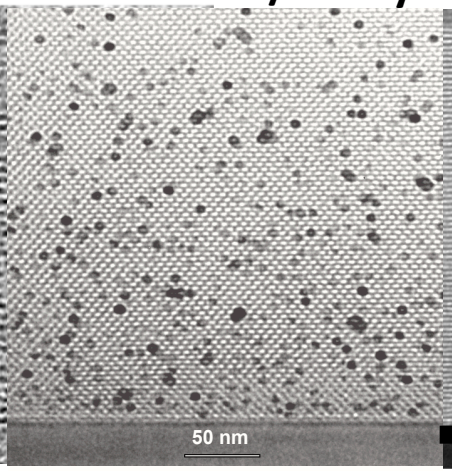
Lu, et al., Nature 1999

Sea-Shell



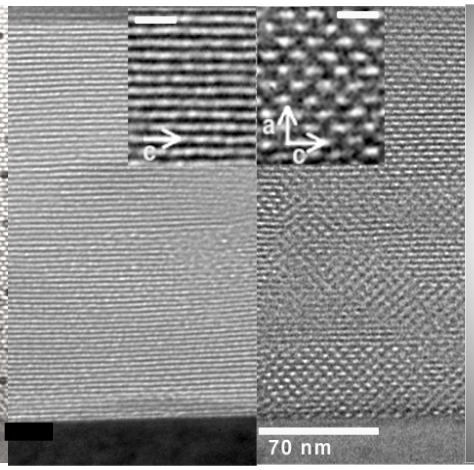
Sellinger et al., Nature 1998

Ag/Silica Antimicrobial/Catalyst



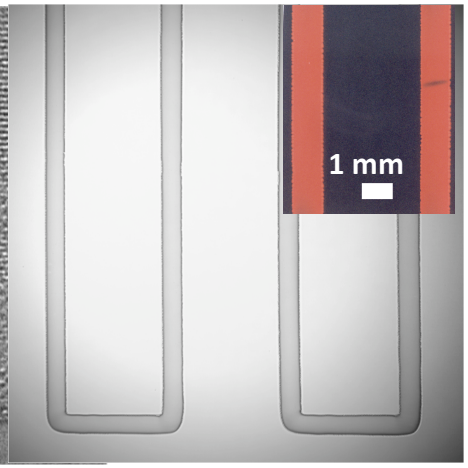
Fan et al., Unpublished

Phase Transition



Doshi et al., Science 2000

Patterns



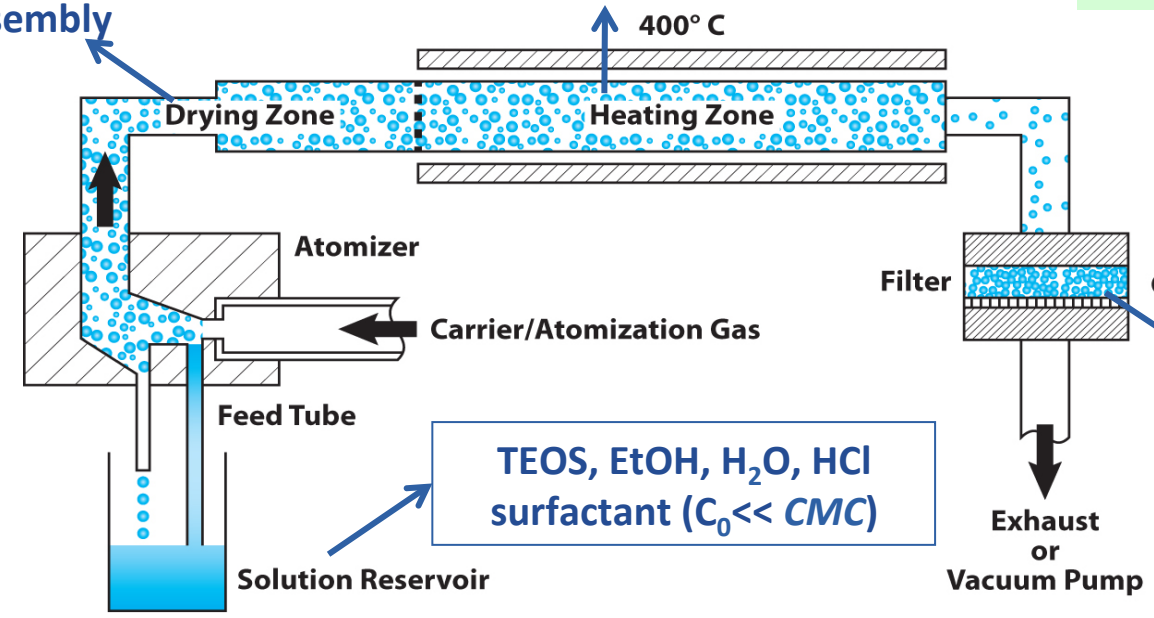
Fan et al., Nature 2000

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

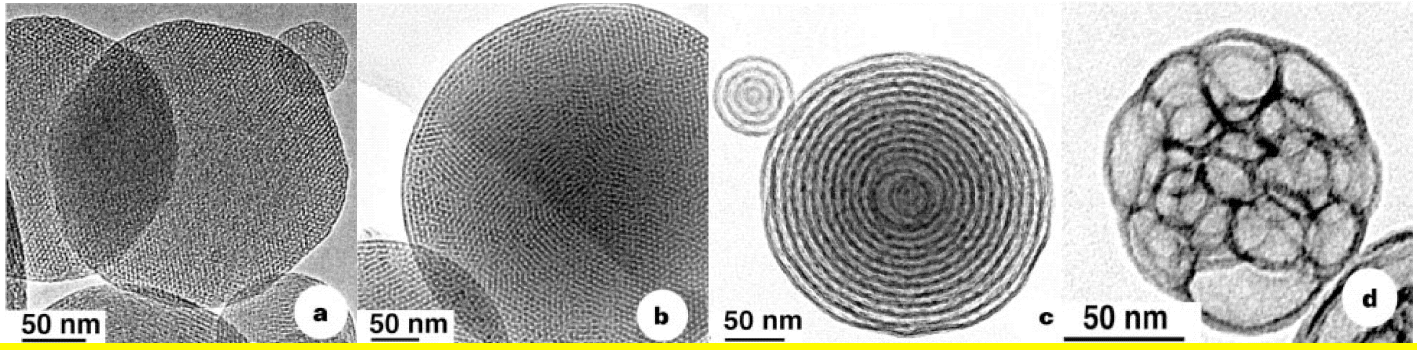
Yunfeng Lu, Brinker et al Nature (1999)



TEOS, EtOH, H_2O, HCl surfactant (C_0<< *CMC*)

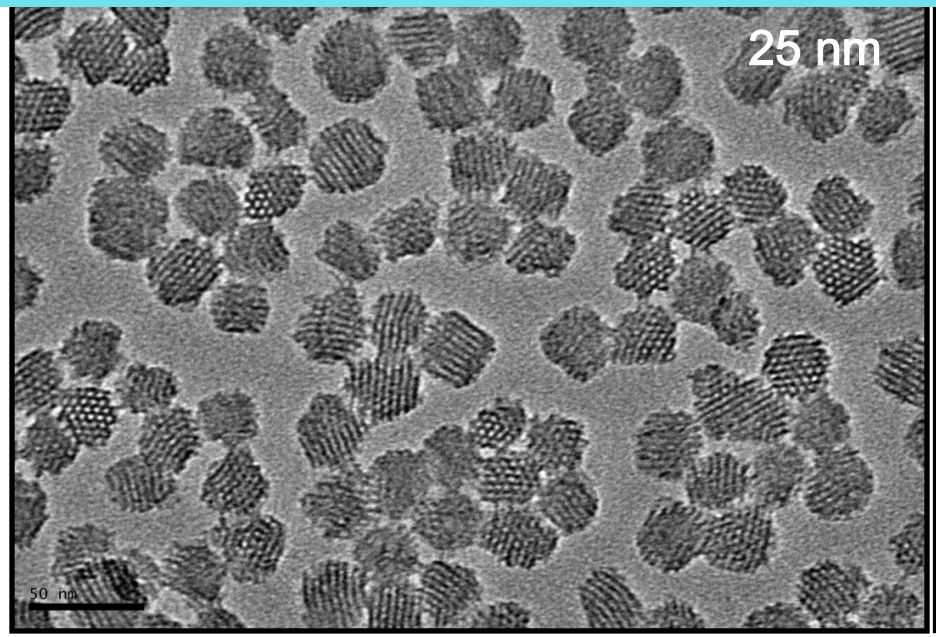
Extract or calcine surfactants to create mesopores

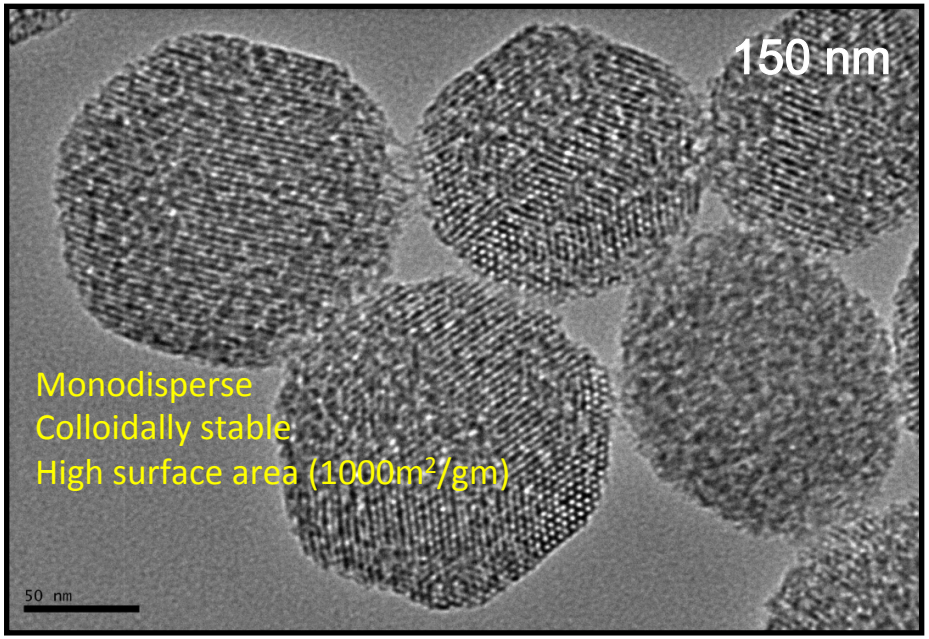
Hexagonal **Cubic** **Lamellar** **Cellular**

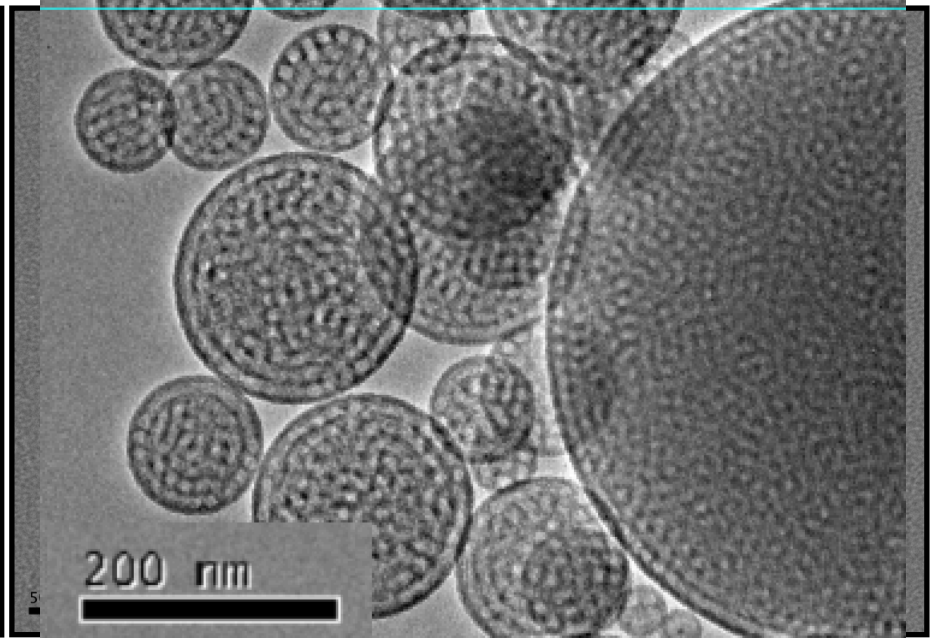


EISA confined to aerosol yields smooth, spherical or faceted mesoporous silica NPs

Second Generation protocell cores are synthesized by Colloidal self-assembly - allows synthesis of monosized silica nanoparticles (MSNPs) needed for directing BD

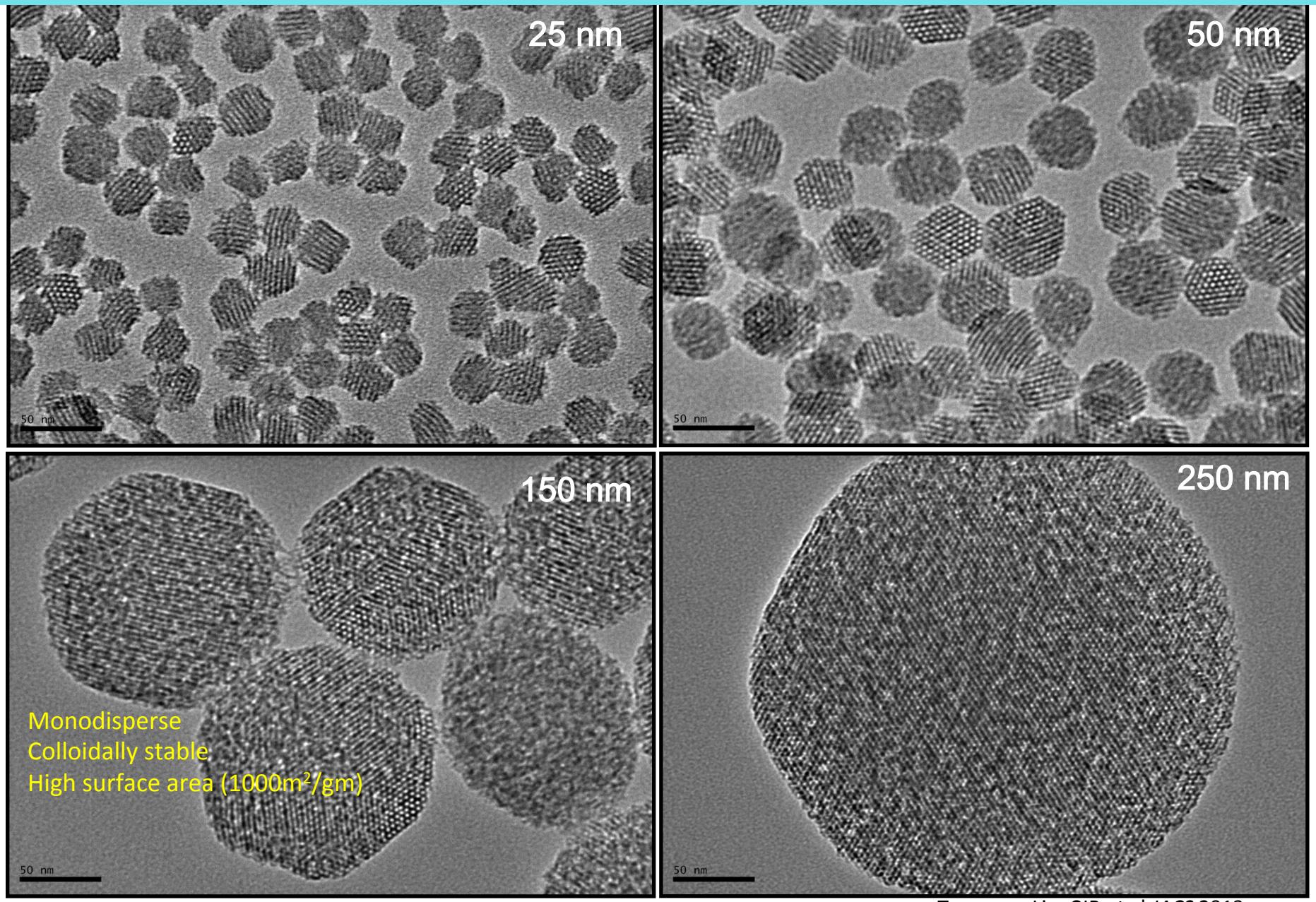






Townson, Lin, CJB et al *JACS* 2013

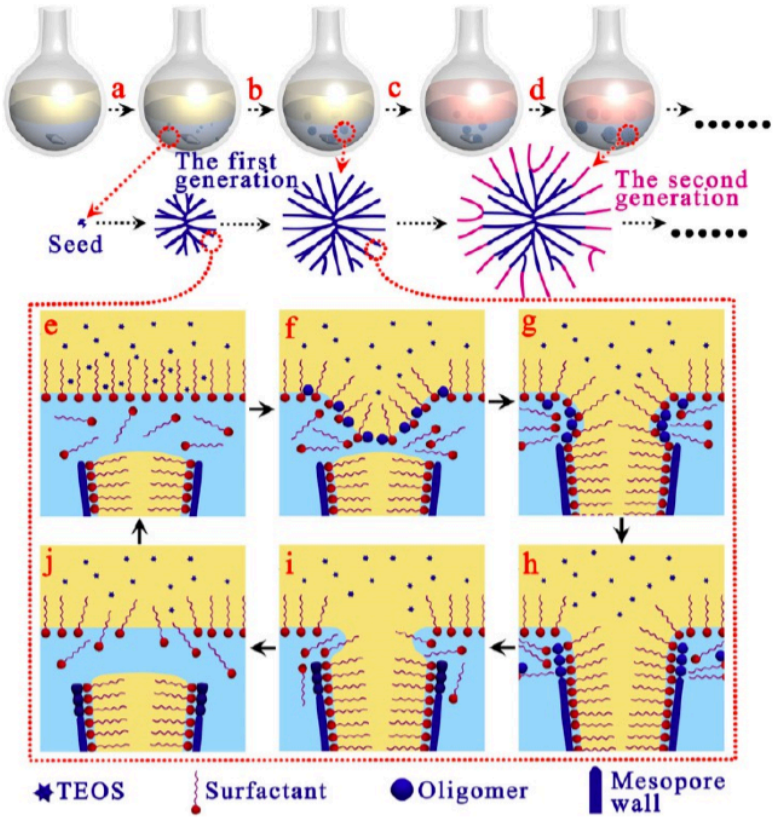
Second Generation protocell cores are synthesized by Colloidal self-assembly - allows synthesis of monosized silica nanoparticles (MSNPs) needed for directing BD



Townson, Lin, CJB et al *JACS* 2013

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[a]

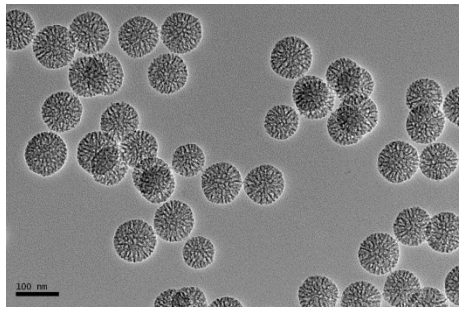


[a](a) Nucleation process of the 3D-dendritic MSNSs; (b) growth process of the first generation of the 3D-dendritic MSNSs; (c) changing the upper oil phase; (d) growth process of the second generation of the 3D-dendritic MSNSs; (e–h) the mechanism of one single mesopore-channel growth with swelling.

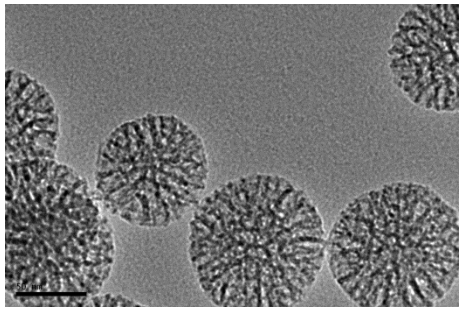
D. Shen et al., Nano Lett. 2014, 14, 923–932

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

13-nm expanded pore MSN



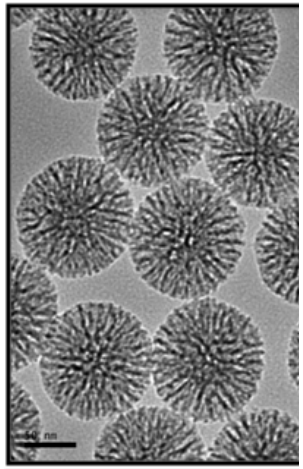
Scale bar: 100 nm

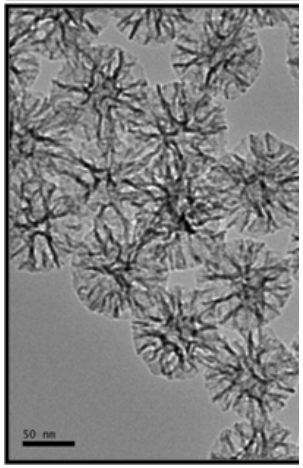


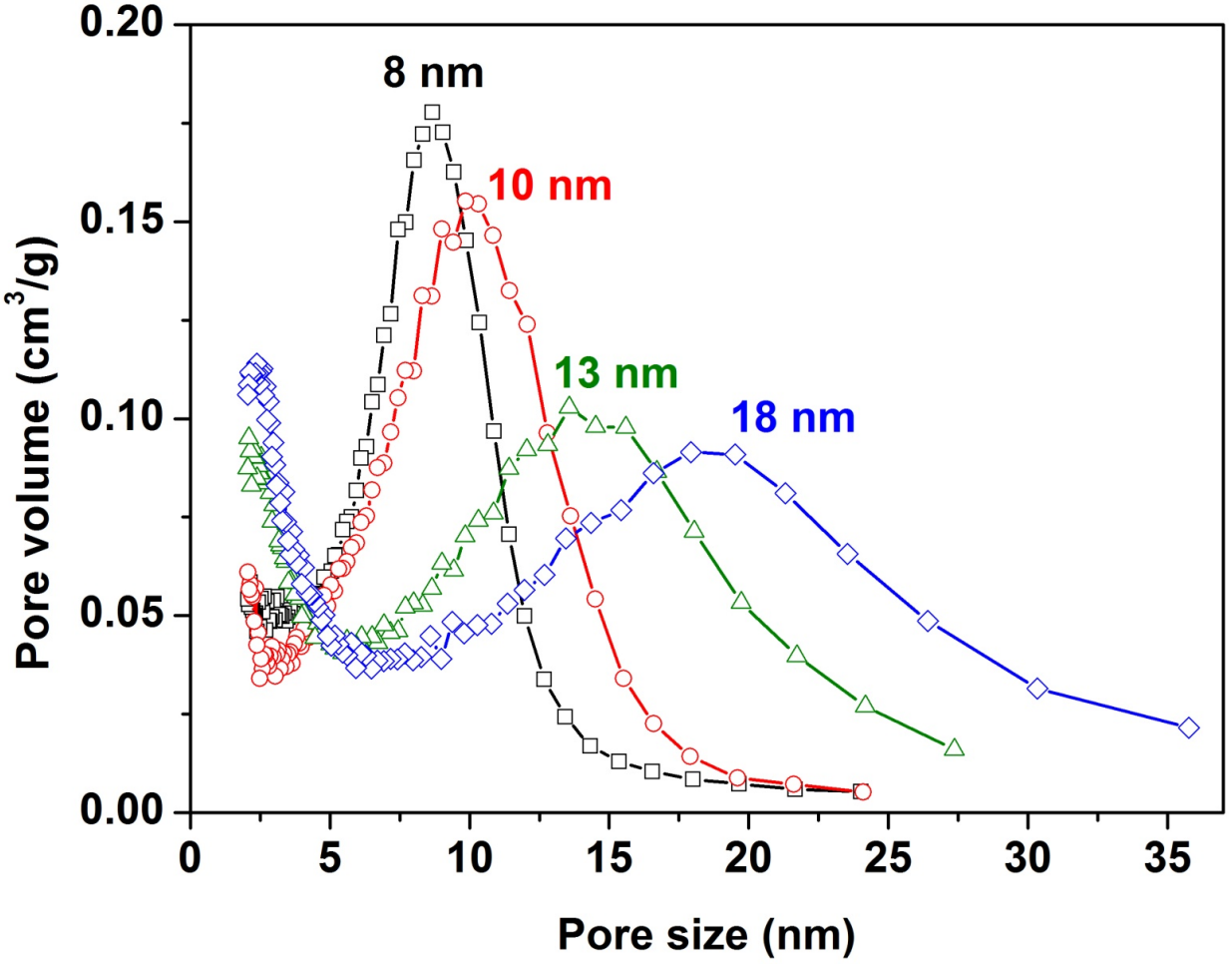
Scale bar: 50 nm

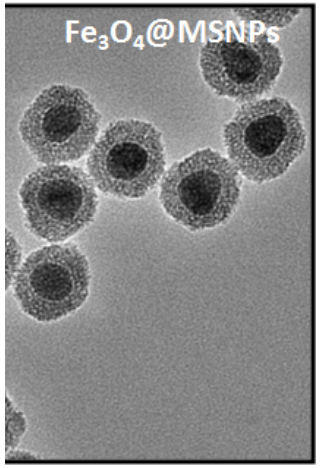
Achraf Noureddine

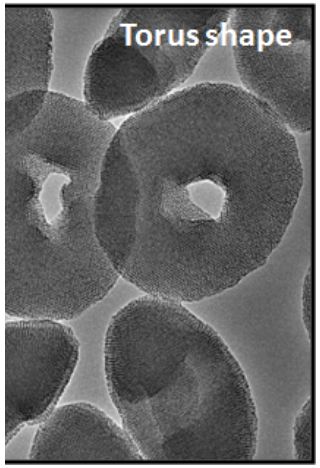
Stable monosized MSNPs can be engineered with varying pore sizes, shapes, and cores to accommodate/package multiple cargo types and direct bio-distribution and internalization





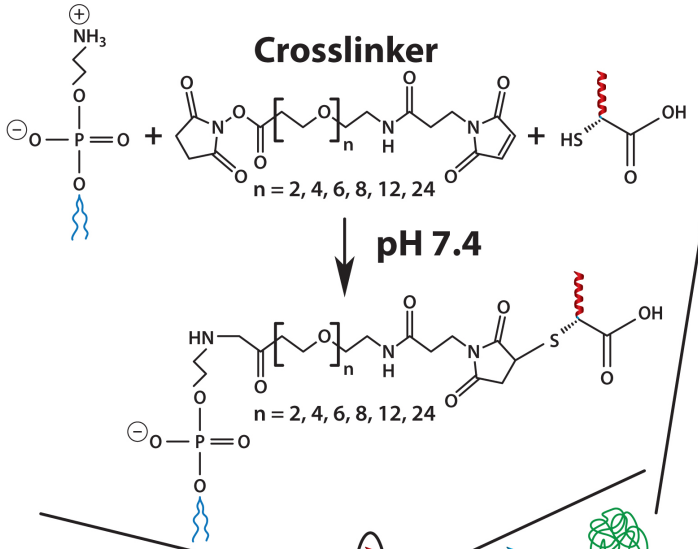






Pores may be large enough for individual plasmid components, e.g Cas9 and gRNA – Plasmids are associated/complexed with MSNP surfaces (Durfee et al. *ACS Nano* 2016)

Supported Lipid Bilayer can be 'tuned' for stability and fluidity



= DOPC T_m = -20°C

or DPPC T_m = 41°C

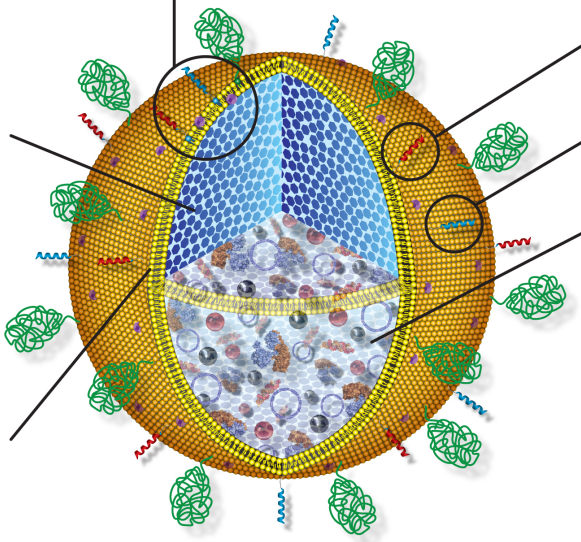
= DOPE (or DPPE)

= 18:1 PEG-2000 PE

= Cholesterol

5%DOPE, 5%PEG, 30% cholesterol, DOPC

Selected against Hep3B by phage display*



Nanoporous Silica Core

Supported Lipid Bilayer

Model system: HCC Hep 3B

Targeting Peptide (SP94)

H_2N-SFSIIHTPILPLGGC-COOH

Endosomolytic Peptide (H5WYG)

H_2N-GLFHAIAHFIHGGWHGLIHGWYGGGC-COOH

Cargo:

Quantum Dot

Nanoparticle

Diphtheria Toxin

siRNA

Plasmid DNA

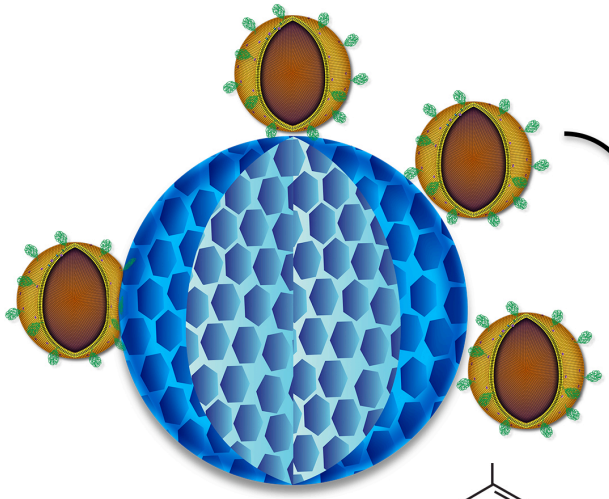
Doxorubicin

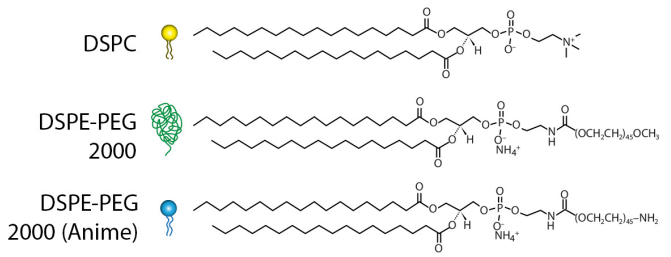
5-Fluorouracil

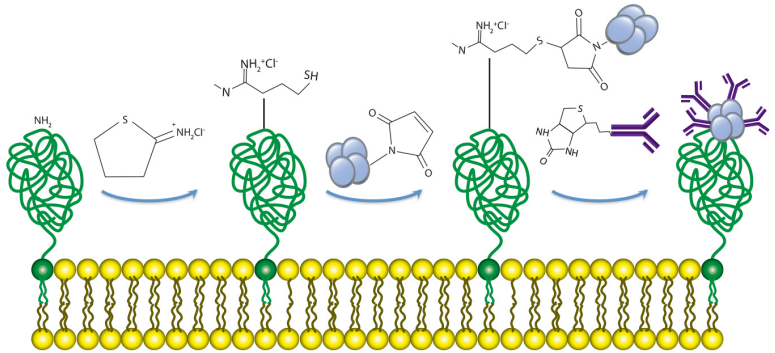
Cisplatin

Molecular Cancer Therapeutics (2008). **7:** 579-589

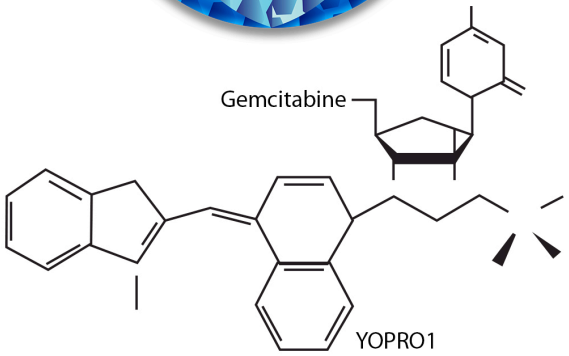
Protocell Formation Occurs by Vesicle Adsorption, Deformation, and Rupture – Fusion is governed by van der Waals and Electrostatic Interactions described by DLVO theory

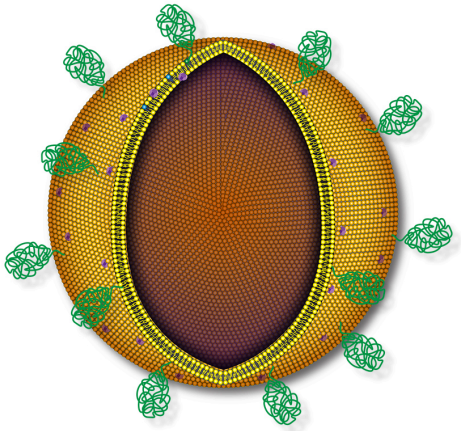


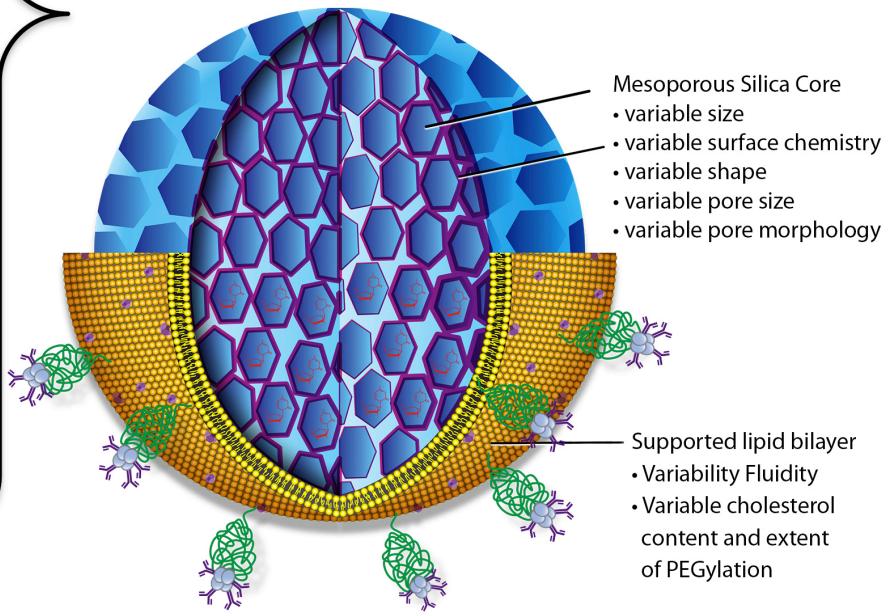




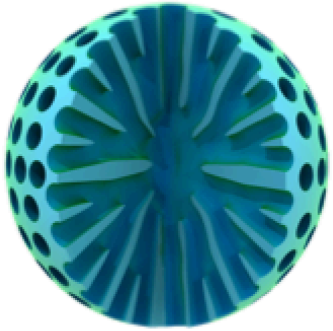
Gemcitabine



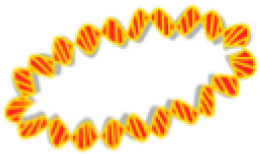




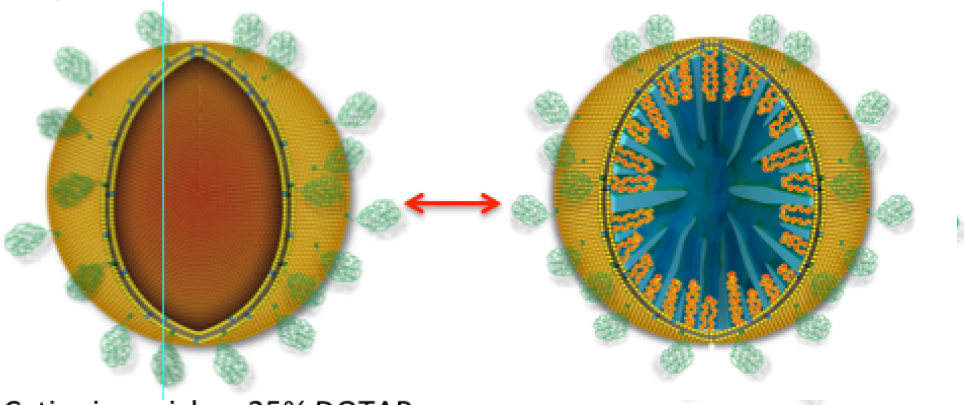
We developed an alternative strategy to load negatively charged nucleic acid and protein cargos in MSNs via cationic vesicle fusion – implications for CRISPR



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

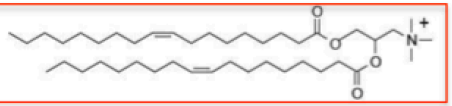


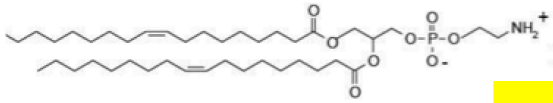
Plasmid – 3-16kb –
Negatively charged

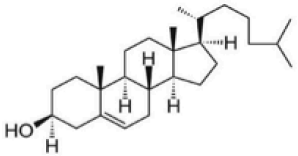


Cationic vesicle > 25% DOTAP

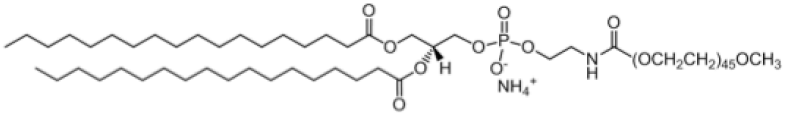
Plasmids packaged via electrostatically mediated assembly
-Spontaneous dissociation/release upon lipid destabilization at low pH

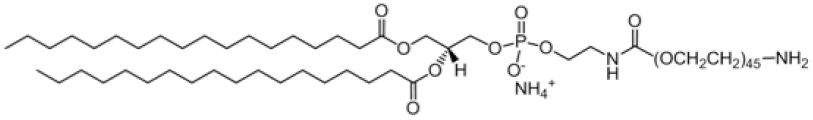
DOTAP 

(Zwitterionic helper lipid) DOPE 

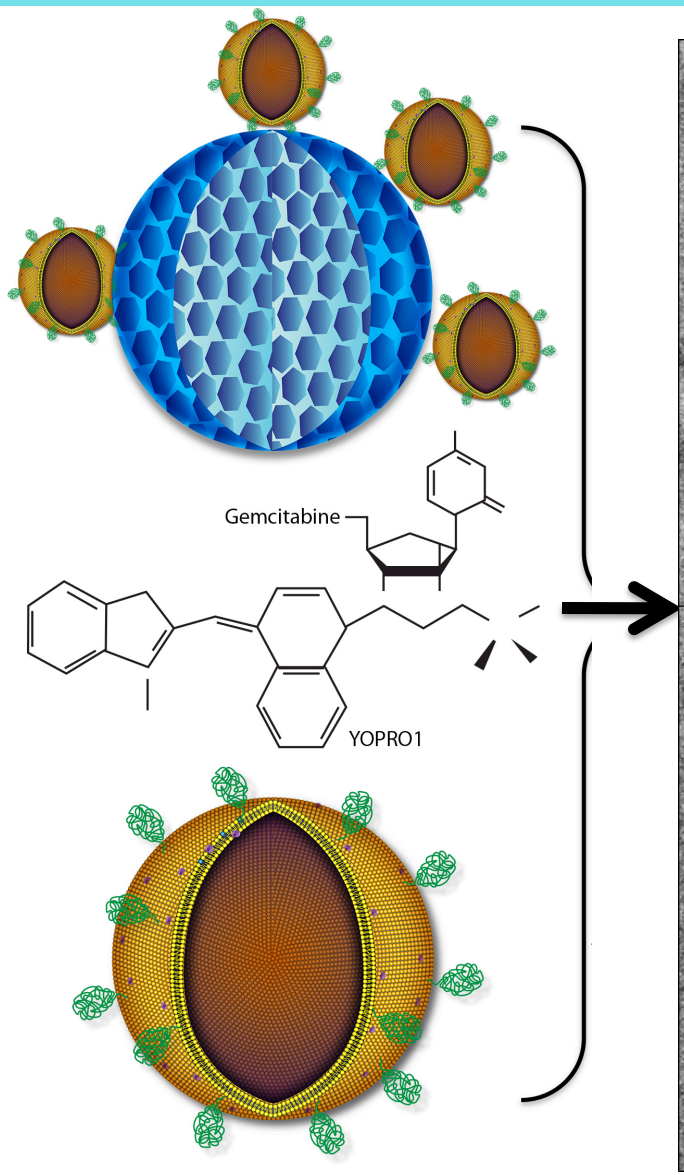
(membrane stability) Cholesterol 

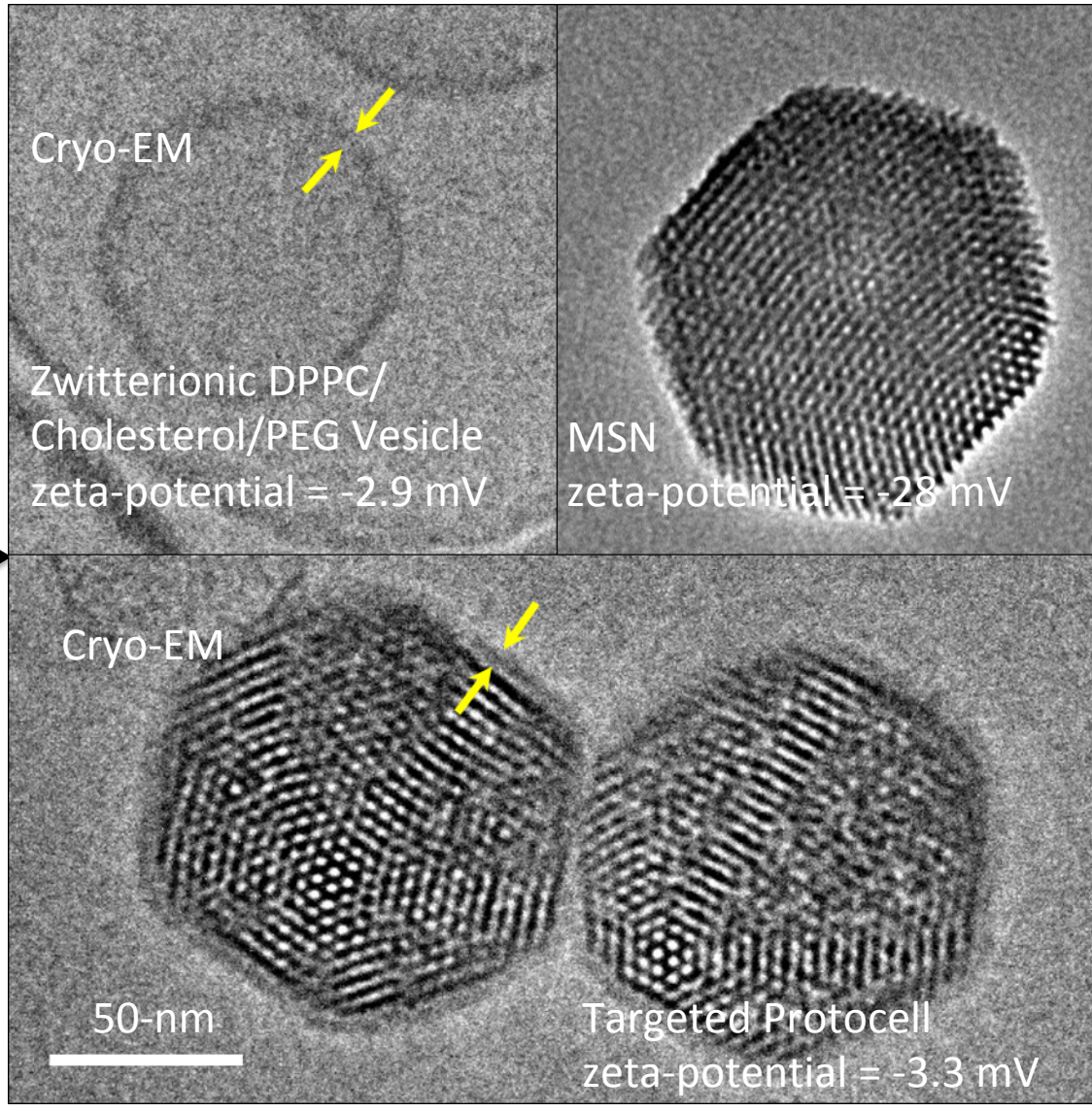
DOTAP
DOTAP/CHOL/DSPE-PEG/DOPE = 47/47/2/4

(in vivo stability) DSPE PEG_{2K} 

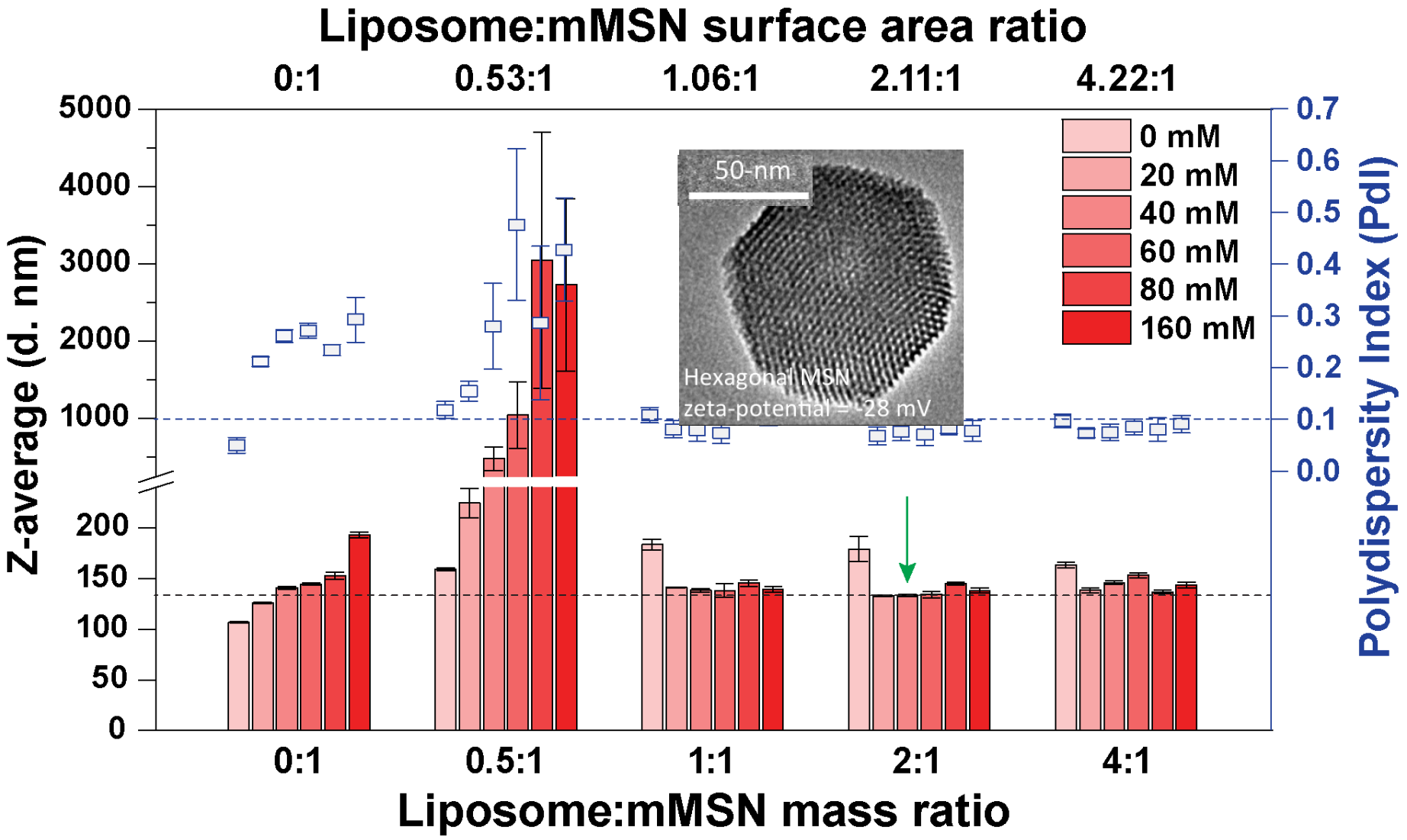
(Targeting) DSPE PEG_{2K} NH_2 

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



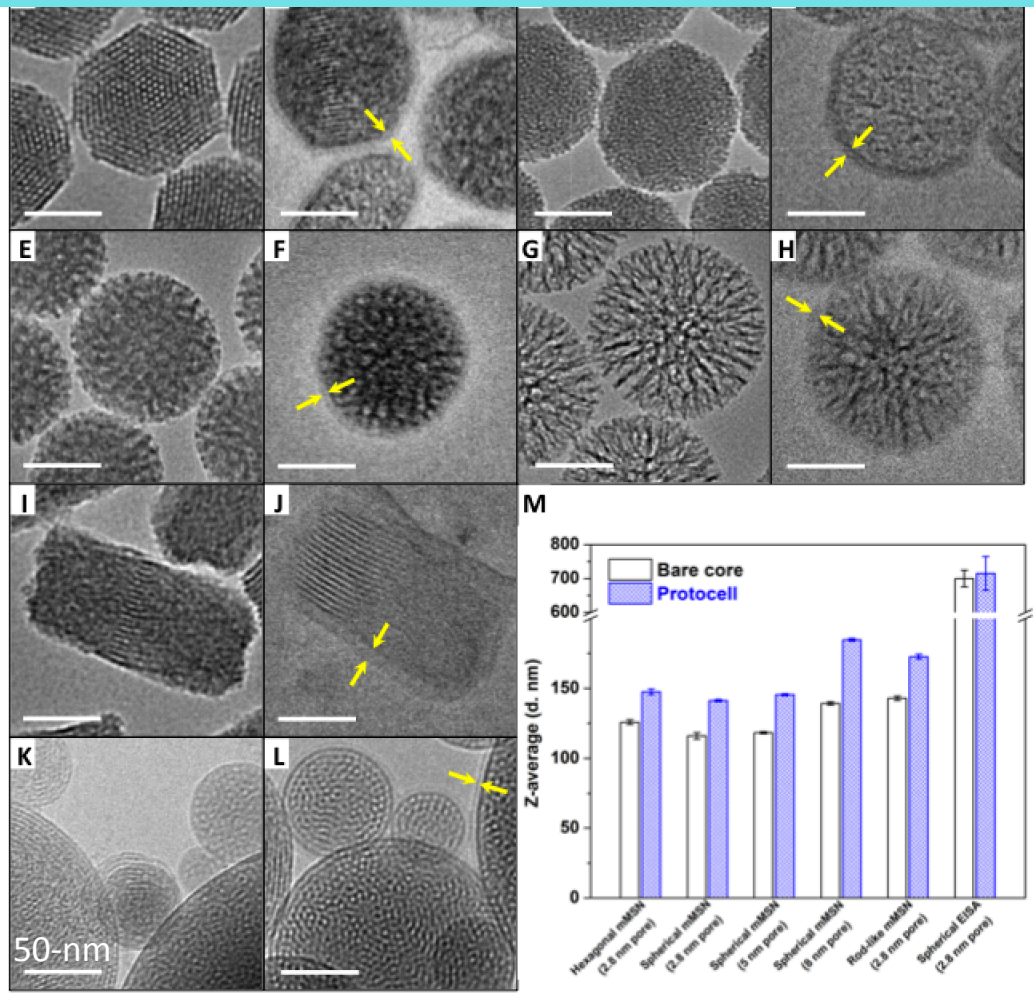


At Neutral pH (PBS buffer) Criteria for Formation of Monosized, Non-Aggregated Protocells are Lipid:MSN Surface Area Ratios > 1:1 and Ionic Strength ≥ 20 mM*



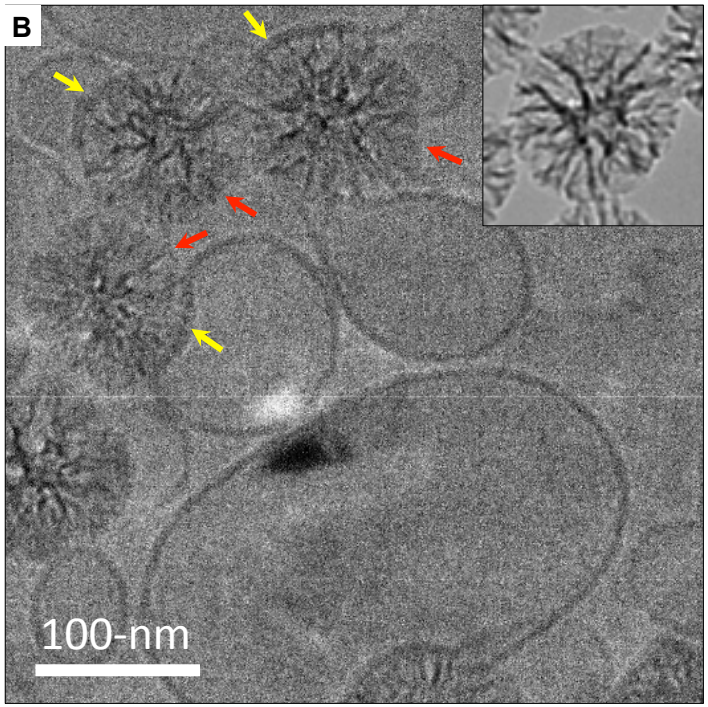
Established robust processing window for monosized protocells: rate of vesicle fusion exceeds greatly rate of MSNP aggregation – trickier for MSNP with larger PSD

The Criteria for Formation of Monosized, Non-Aggregated Protocells are Lipid:MSN Surface Area Ratios Exceeding 1:1 and Ionic Strength > 0 mM

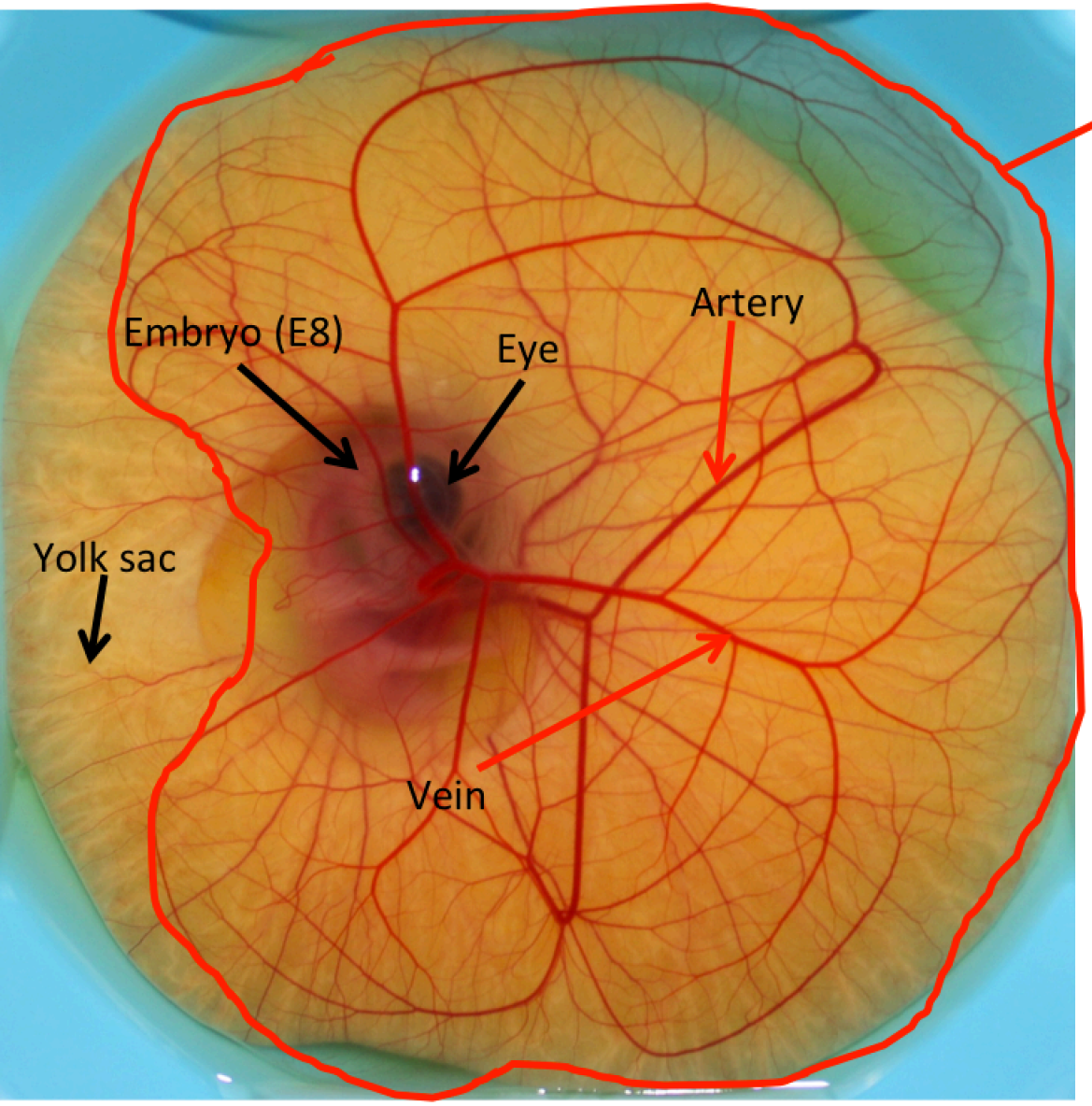


Sample	Hydrodynamic core diameter (nm)	Hydrodynamic protocell diameter (nm)
Hexagonal (2.8 nm pore)	125.87 ± 1.70	147.53 ± 2.02
Spherical (2.8 nm pore)	116.07 ± 2.35	141.30 ± 0.75
Spherical (5 nm pore)	118.33 ± 0.76	145.50 ± 0.62
Spherical (8 nm pore)	139.23 ± 1.15	184.70 ± 1.06
Rod-like (2.8 nm pore)	142.93 ± 1.53	172.67 ± 1.72
Spherical EISA (2.8 nm pore)	700.00 ± 24.68	715.20 ± 49.79

- 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?



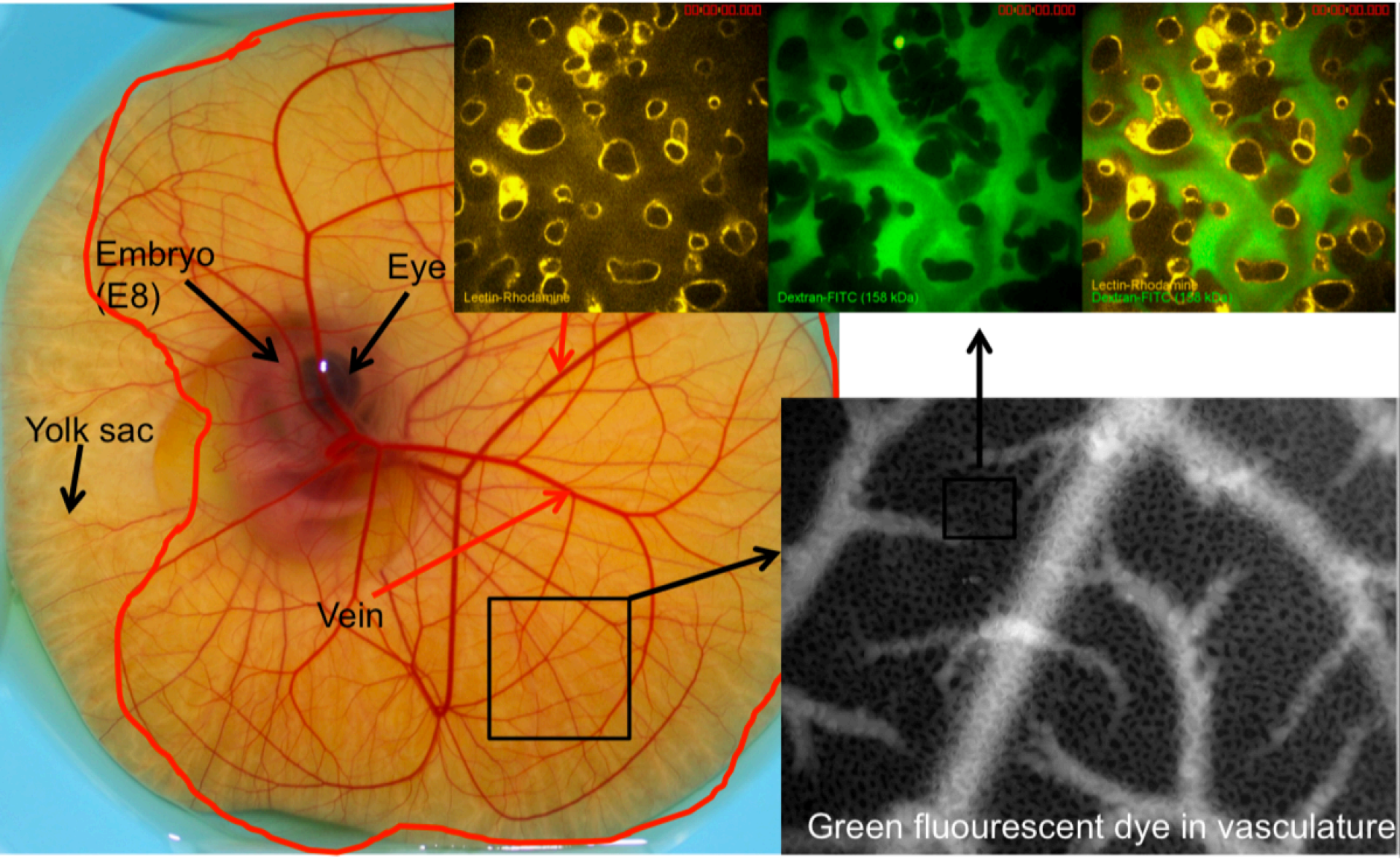
How do we rapidly assess in vivo colloidal stability and targeting specificity of NPs in a relevant model system?

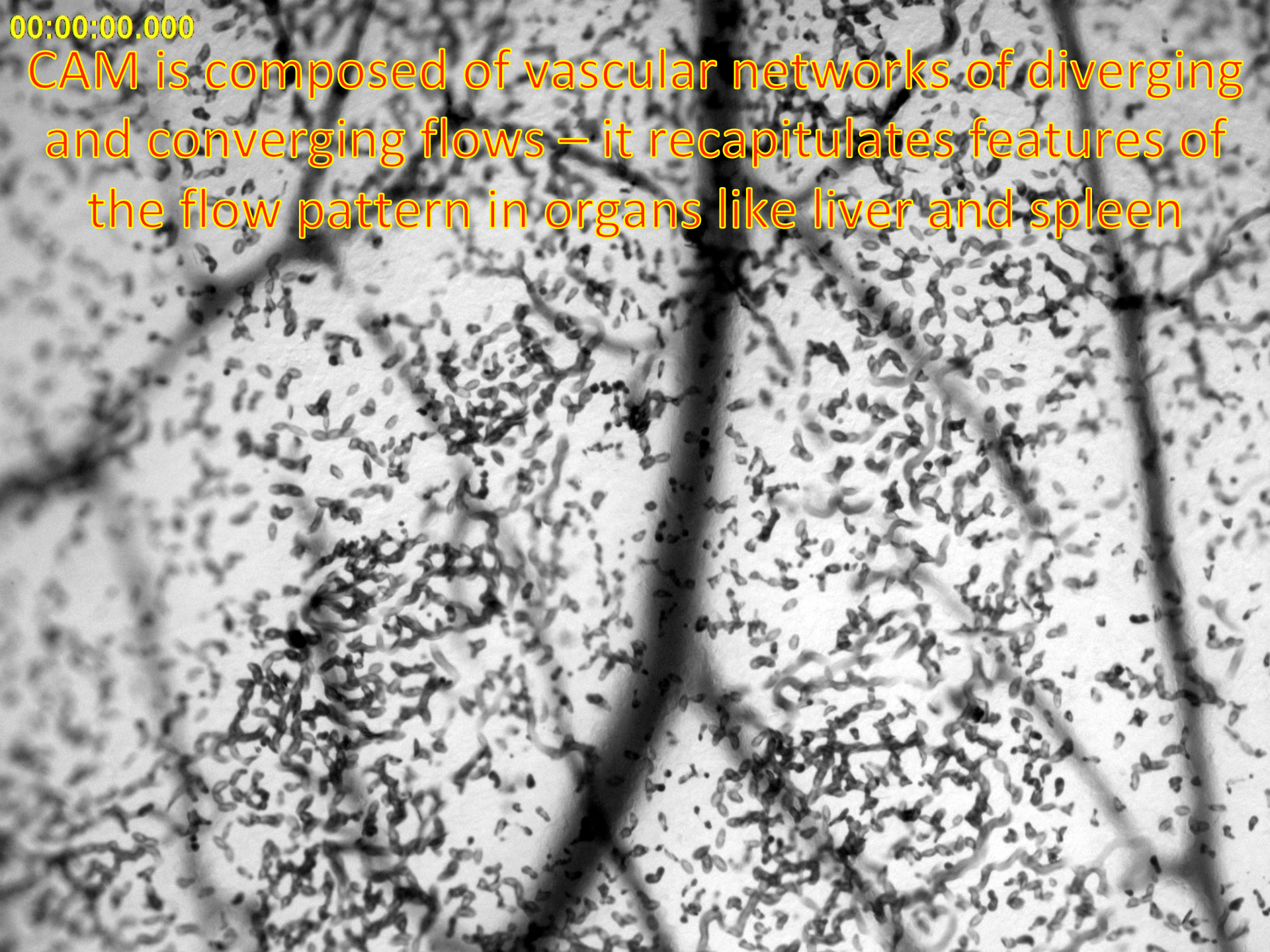


Collaboration with Hon Sing Leong now @ Mayo Clinic

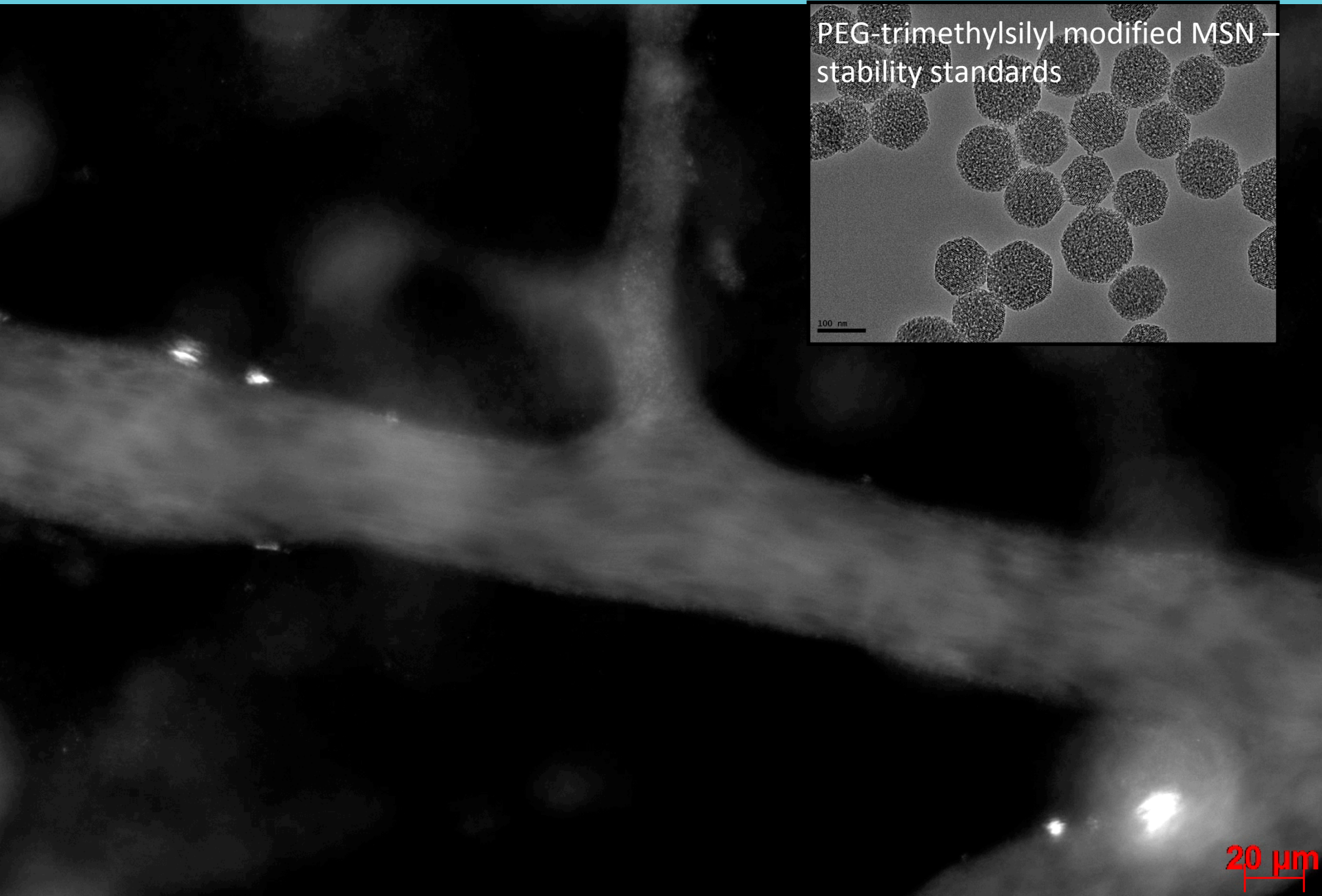
Chick Chorioallantoic Membrane (CAM) *Ex ovo* Avian Embryo Model -

serves as accessible model system in which to examine NP stability and targeting in a complex biologically relevant medium

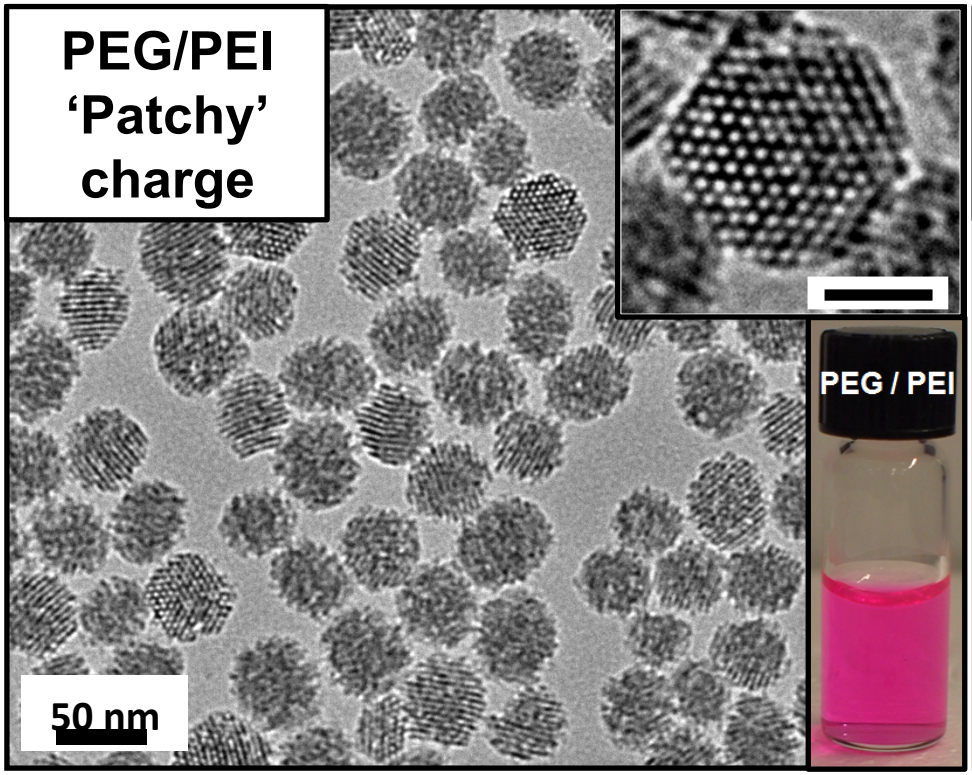


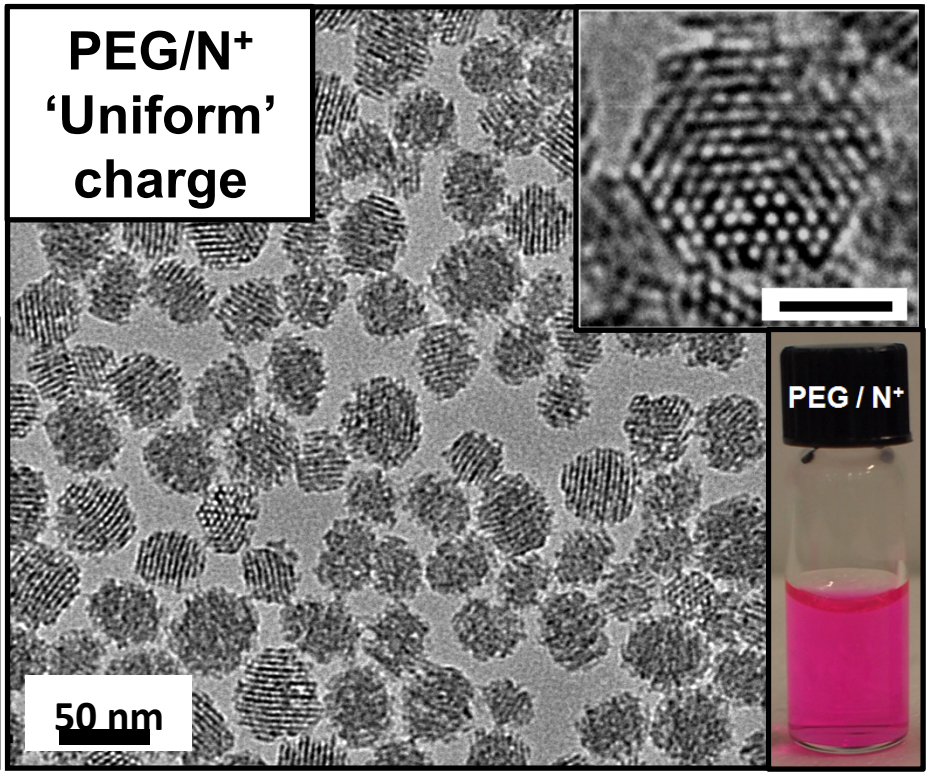

00:00:00.000
CAM is composed of vascular networks of diverging and converging flows – it recapitulates features of the flow pattern in organs like liver and spleen

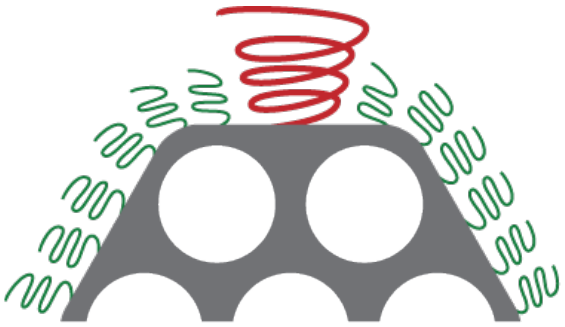
‘Hydrodynamics’ of monodisperse, stable PEG/TMS MSNPs in arterial network of CAM – *Instant Gratification* for establishing NP structure / property relationships



Synthetic Design of Size, Charge, and Colloidal Stability Matched MSNPs







: PEI

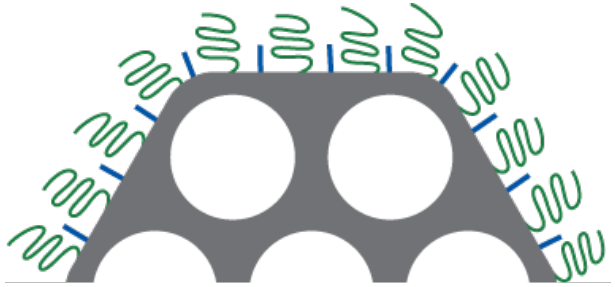
: PEG

Synthetic condition: PEI/ PEG = 1/14

PEG-silane Mw: 550-750

PEI-silane Mw: 1500-1800

N^+-silane Mw: 258

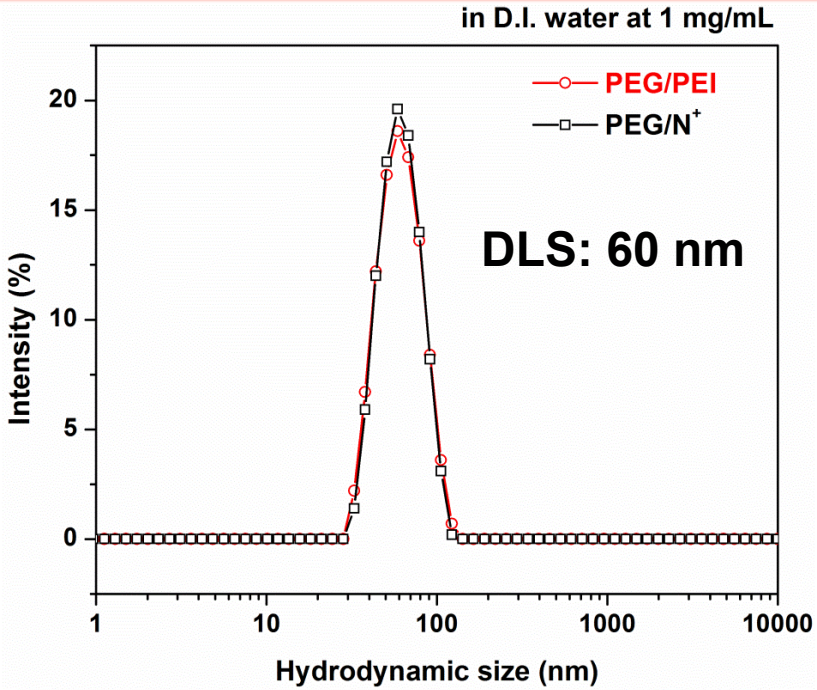


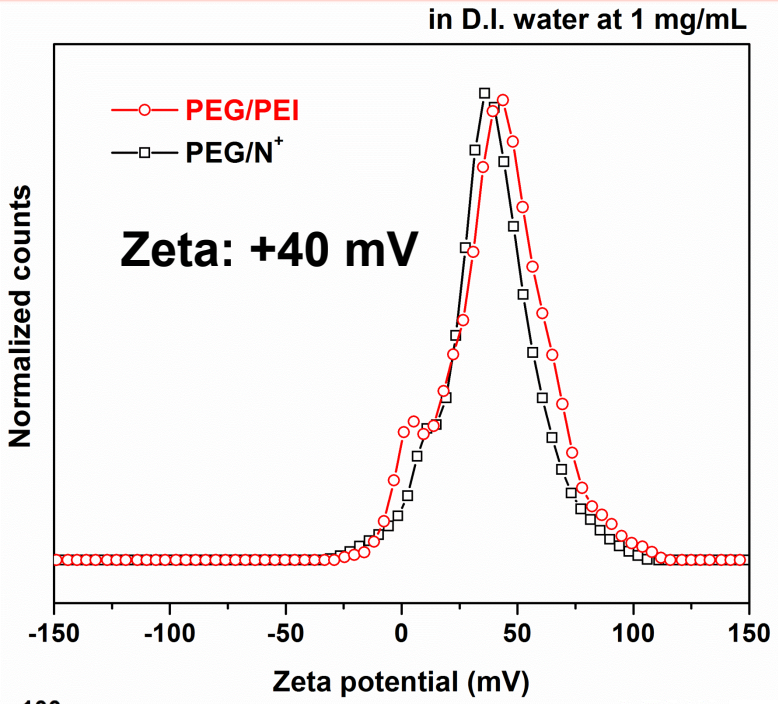
: Quaternary amine

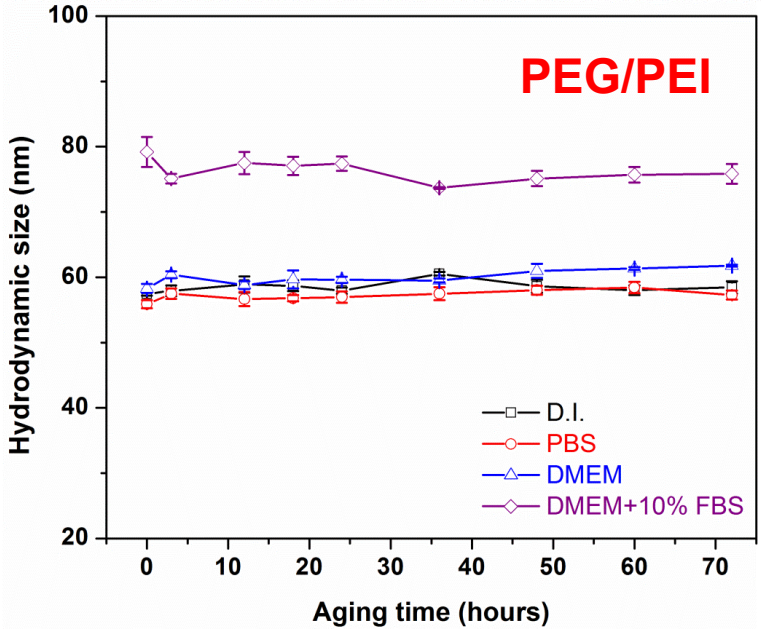
: PEG

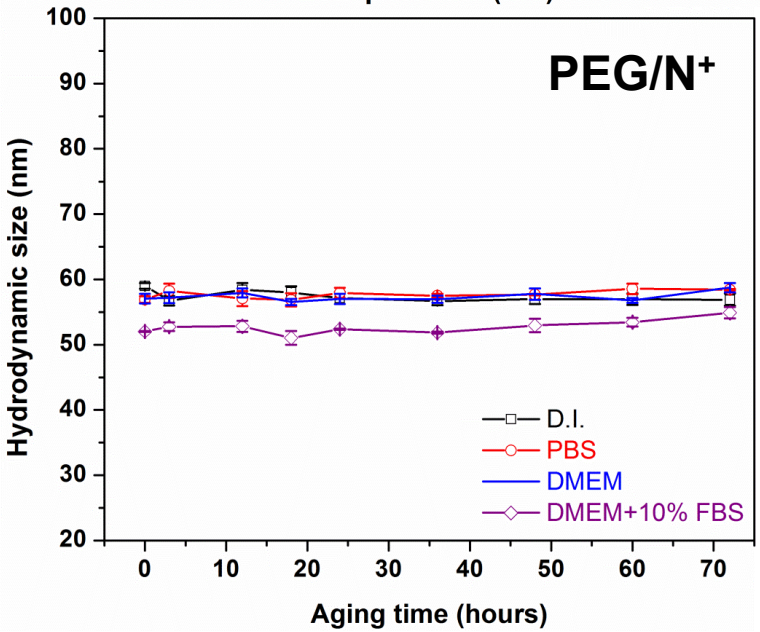
Synthetic condition: N^+ / PEG = 1/1.7

Characterization-DLS, Zeta, and Long-Term Colloidal Stability

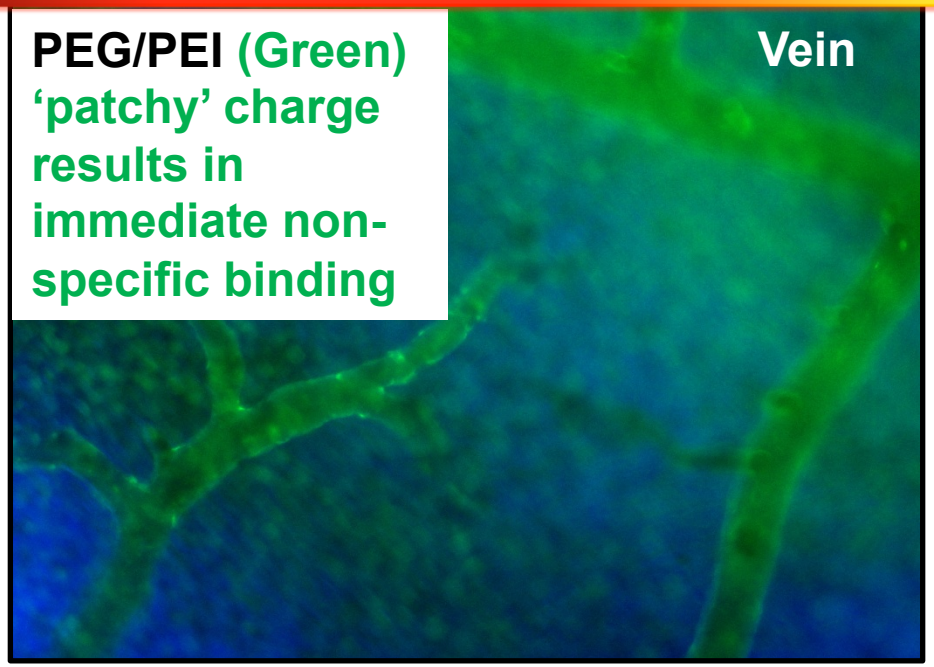


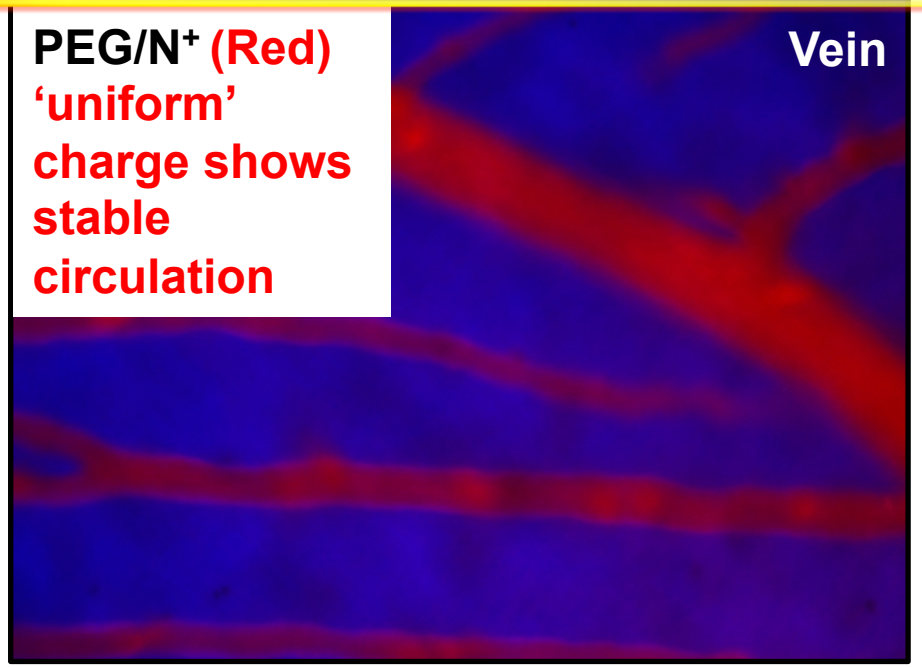


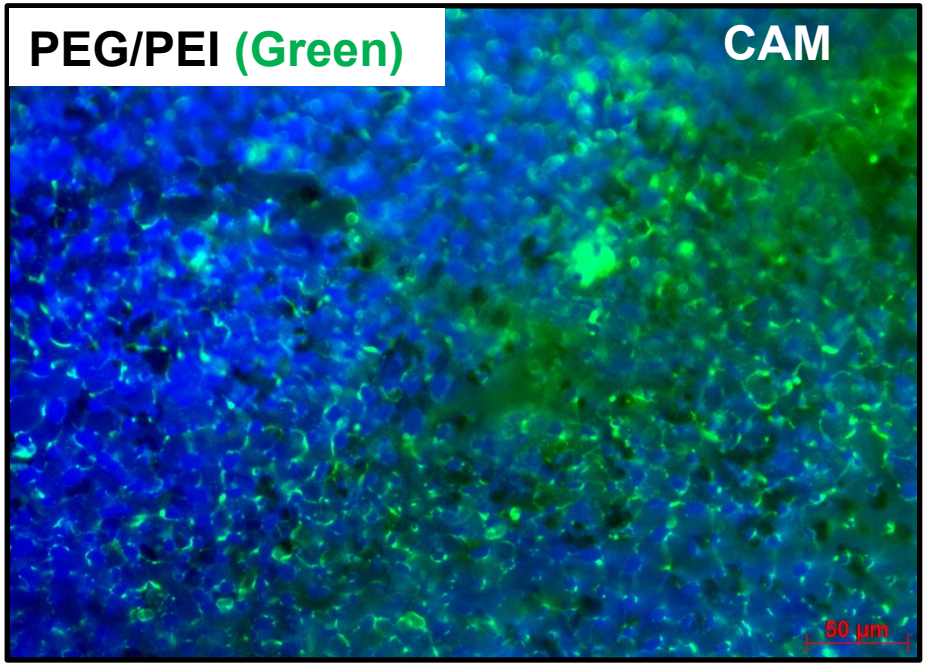


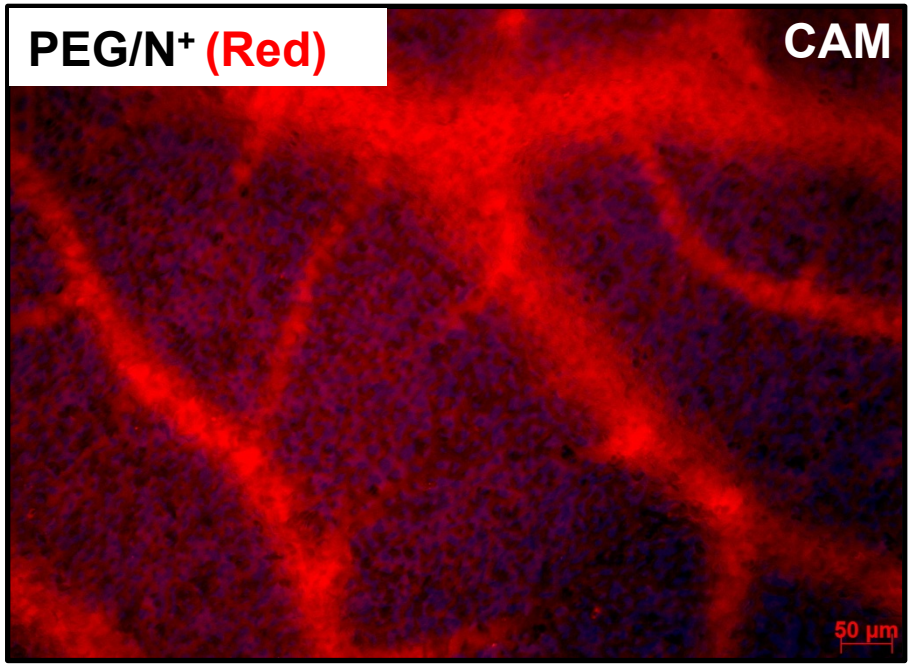


In Vivo Binding Difference-PEG/PEI and PEG/N^+

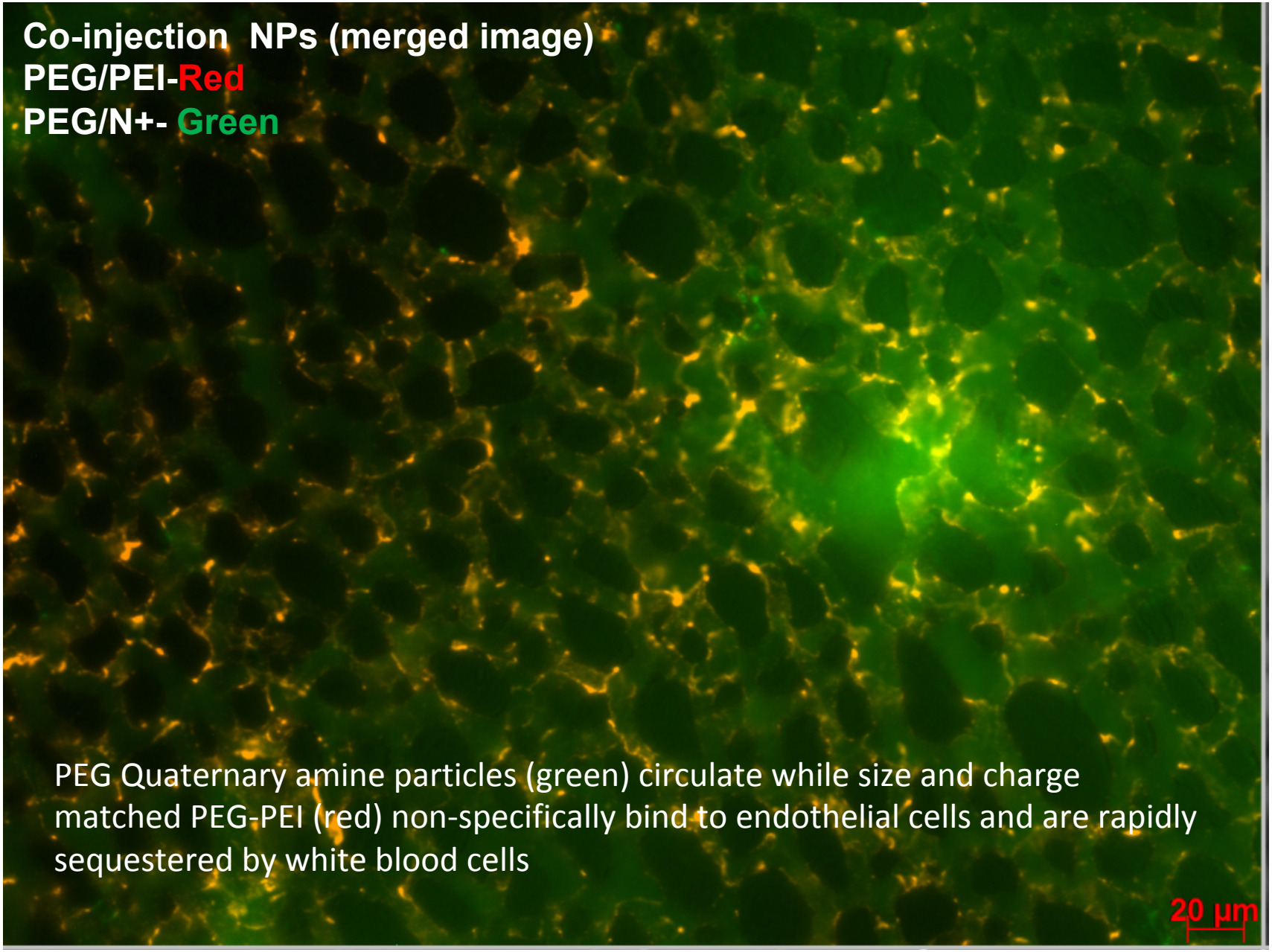




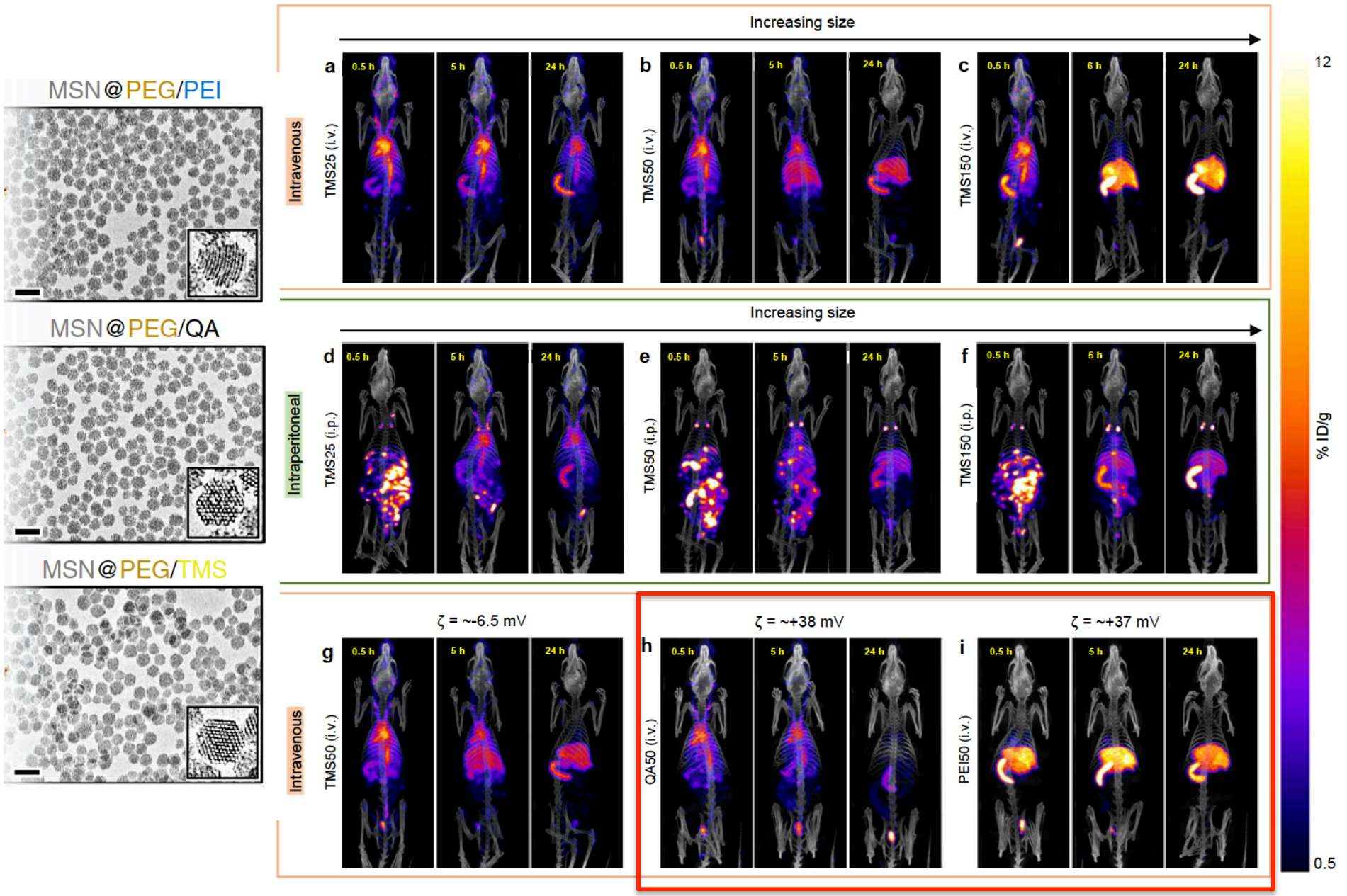




In Vivo Binding Difference-PEG/PEI and PEG/N^{+}(co-injection)



CAM results verified in vivo in Rat Model using ^{111}In SPECT Imaging



Single photon emission computed tomography

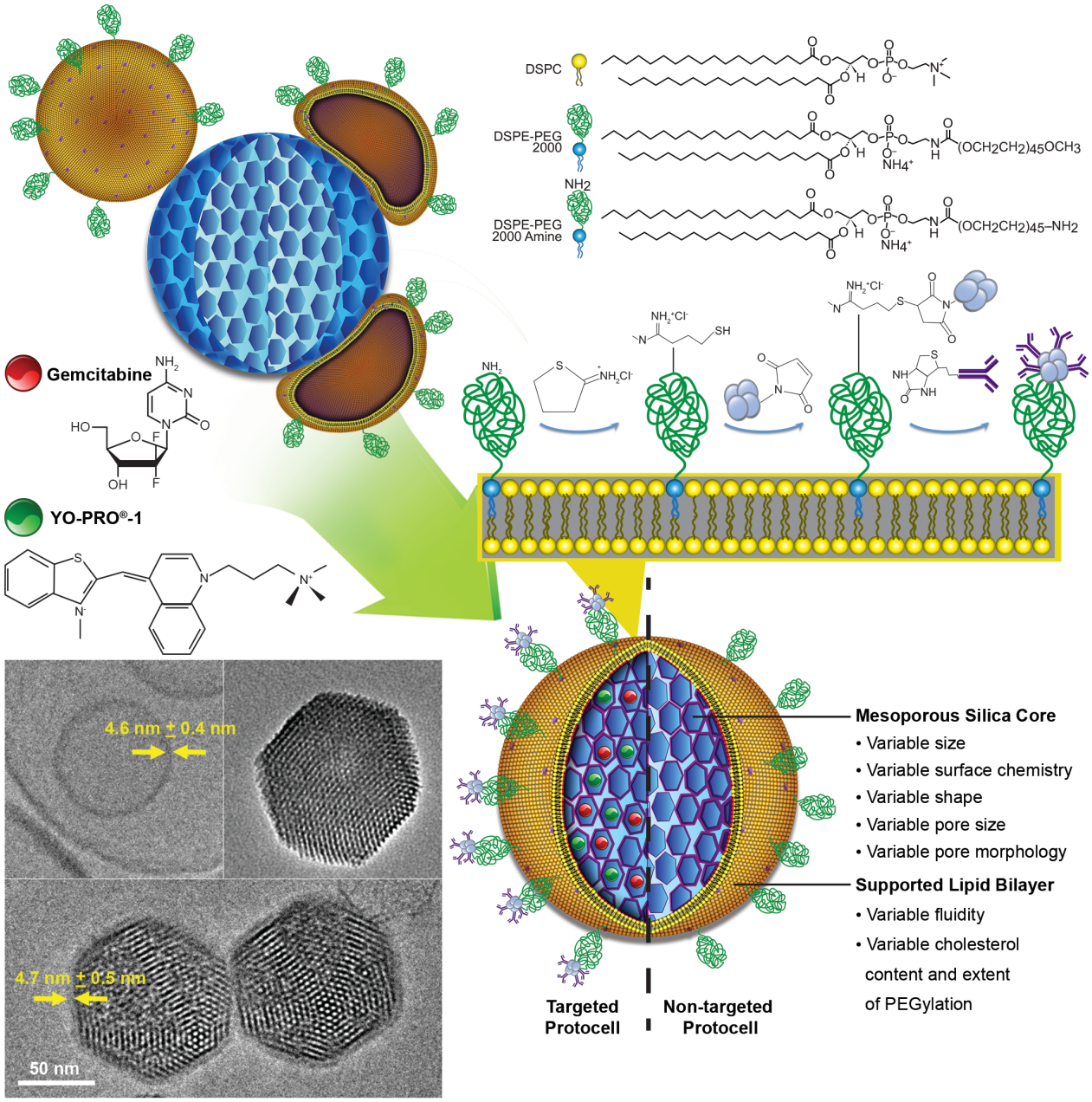
Dogra, Butler, Brinker et al *Nature Communications, 2018*

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

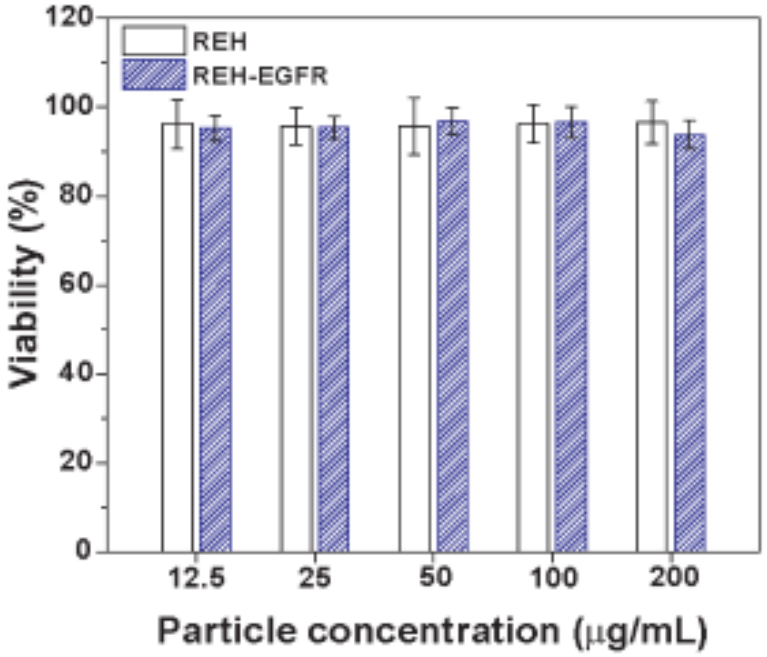
Scheme 1 – Schematic depicting lipid vesicle fusion onto nanoparticles to form mesoporous silica-supported lipid bilayer nanoparticles (protocells). Drug (gemcitabine) and/or fluorescent molecular cargo (YO-PRO®-1) loaded protocells were assembled by soaking nanoparticle cores with cargo for 24h in aqueous 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.



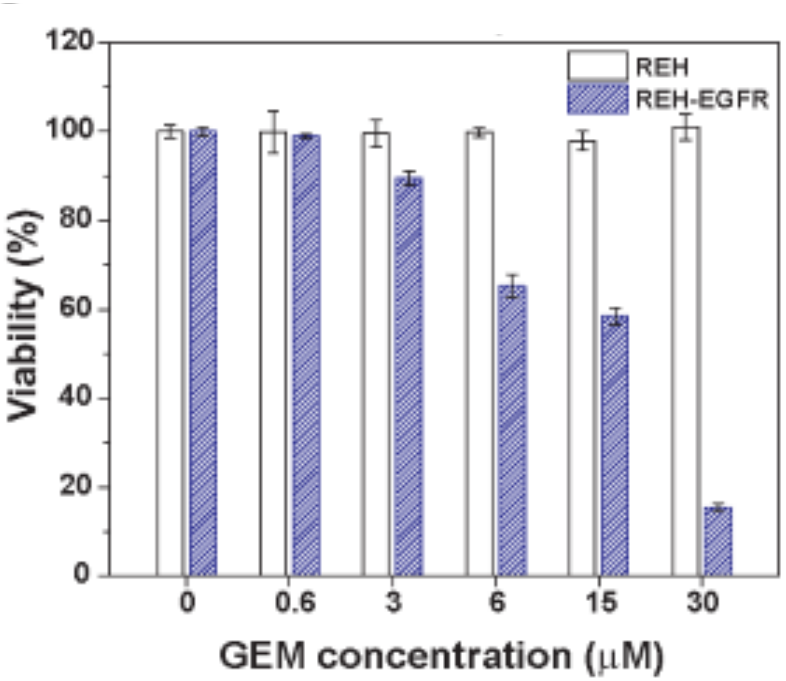
Durfee et al *ACS Nano*, 2016

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

Unloaded Protocells



GEM loaded Protocells



Durfee *et al.*, *ACS Nano 2016*

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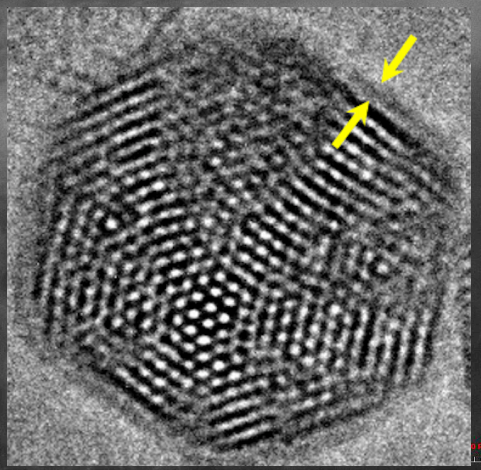
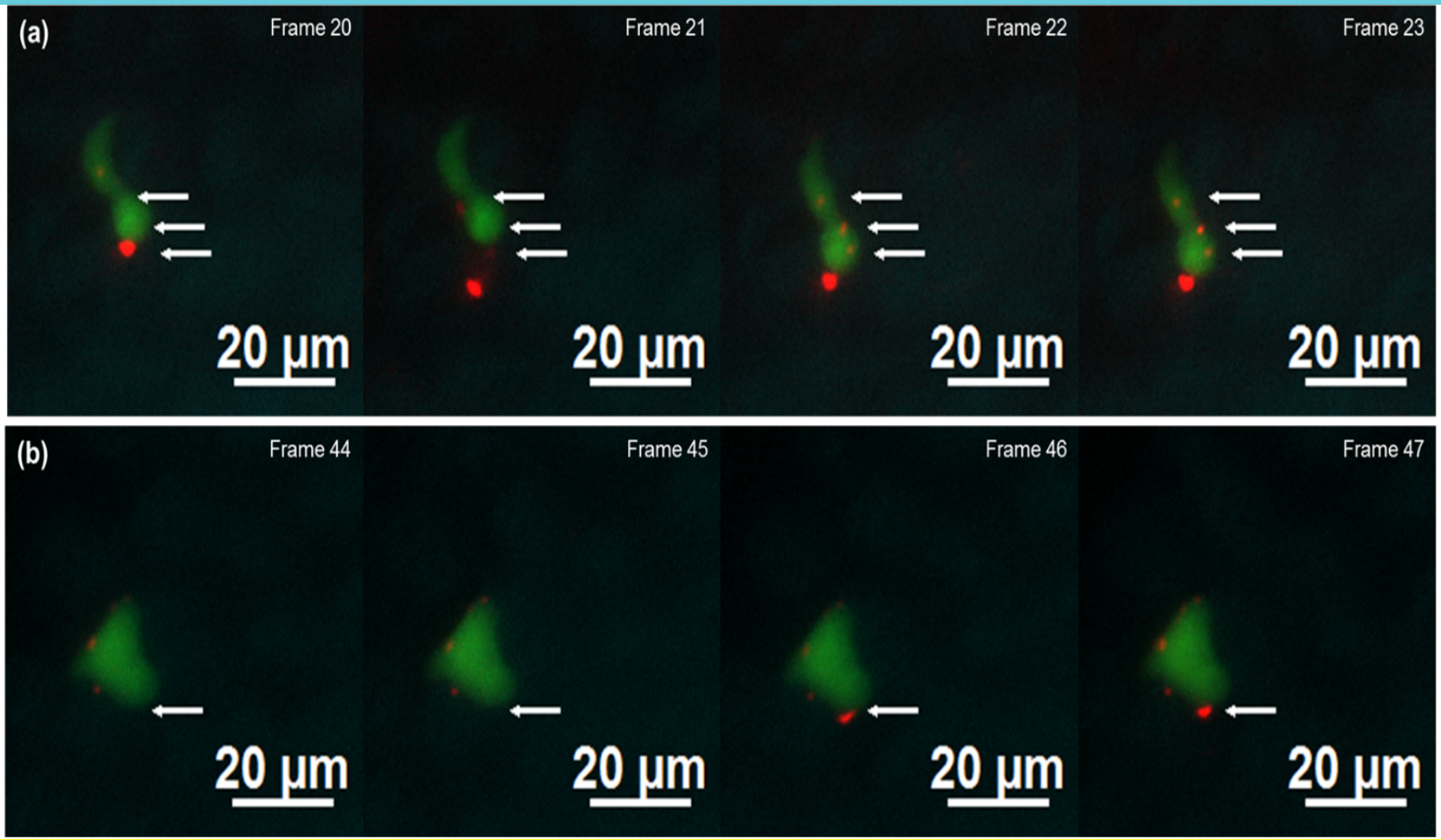
CAM imaging of loaded ~110-nm Ab-Targeted Protocells 30- minutes post-injection - shows circulation with no apparent non-specific binding or uptake by white blood cells



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)

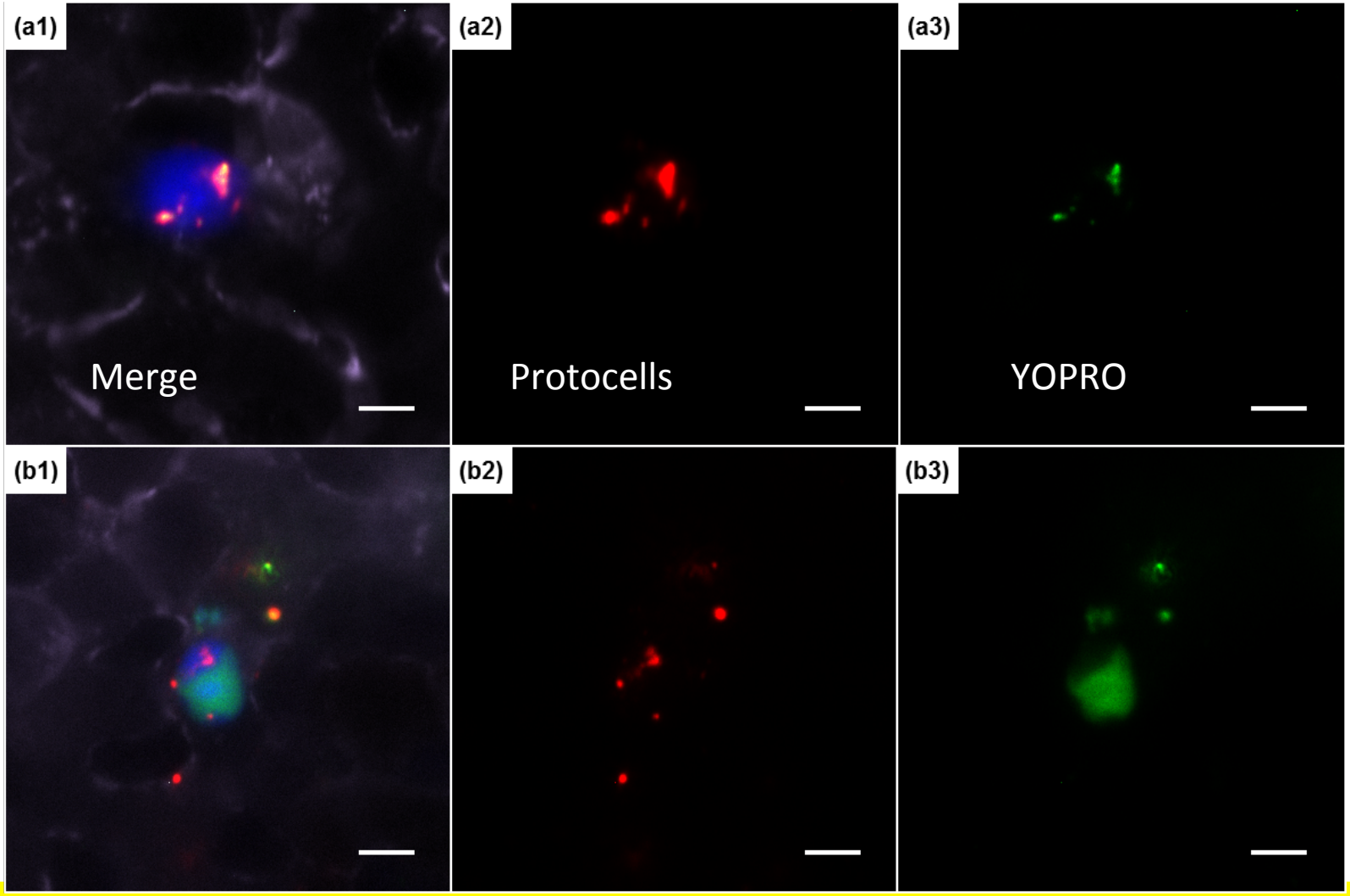


EGFR positive REH leukemia cells (green) are rapidly targeted (<5mins) by EGFR-antibody targeted monosized protocells (red). 30 Frames per second. *ACS Nano 2016*

Using intravital imaging in the CAM we visualized protocell binding and intracellular delivery of a cell impermeant drug surrogate (YOPRO)

4 hours post injection

16 hours post injection

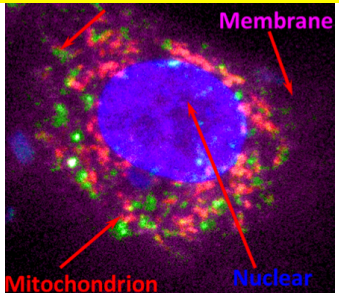


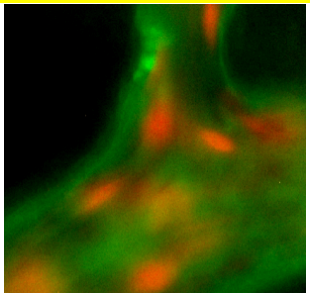
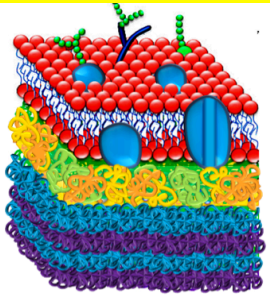
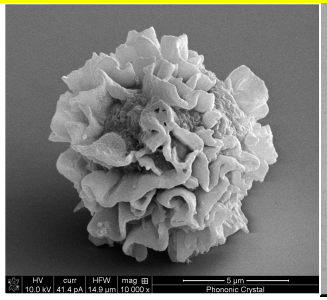
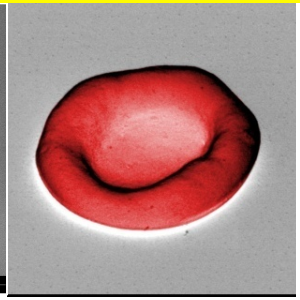
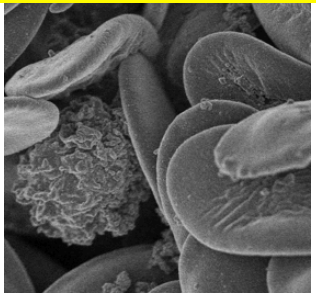
EGFR positive leukemia cells (blue) show retention of targeted protocells (red) but no delivery of YOPRO cargo (green) at 4hrs. However by 16 hours, targeted cells show intra cellular delivery of cargo.

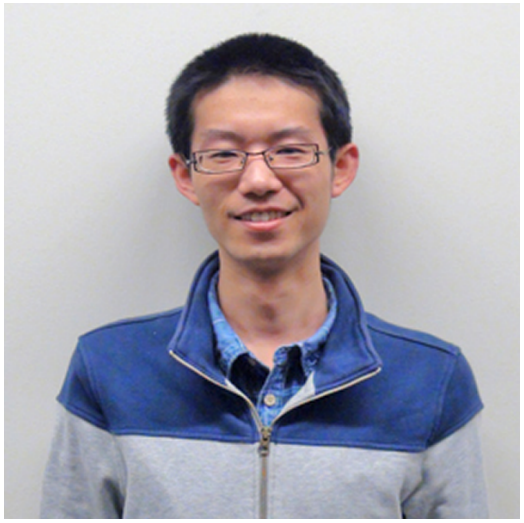
Durfee et al *ACS Nano* 2016: Current study being conducted at Children's Hospital of Philadelphia

Theme 2: Biomimetic Rebuilding of Multifunctional Red Blood Cells: Modular Design Using Functional Components

FNANO Virtual Meeting May 5, 2020



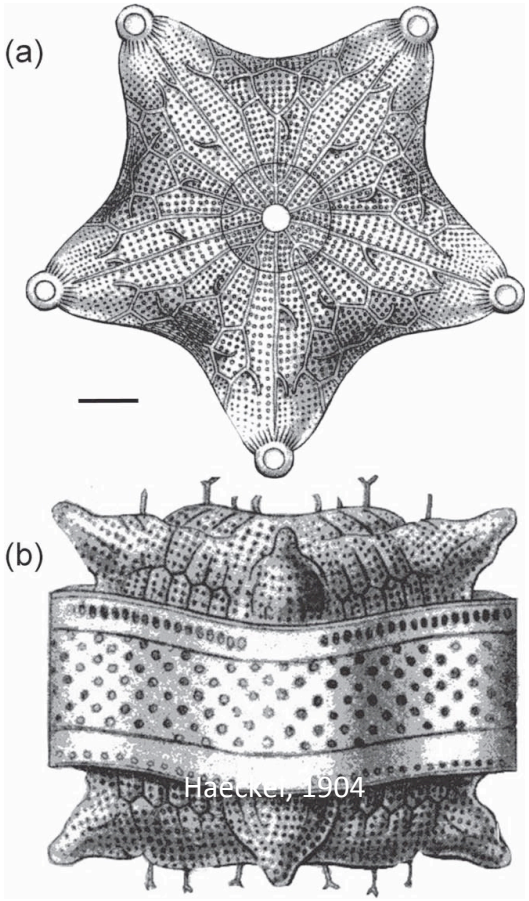


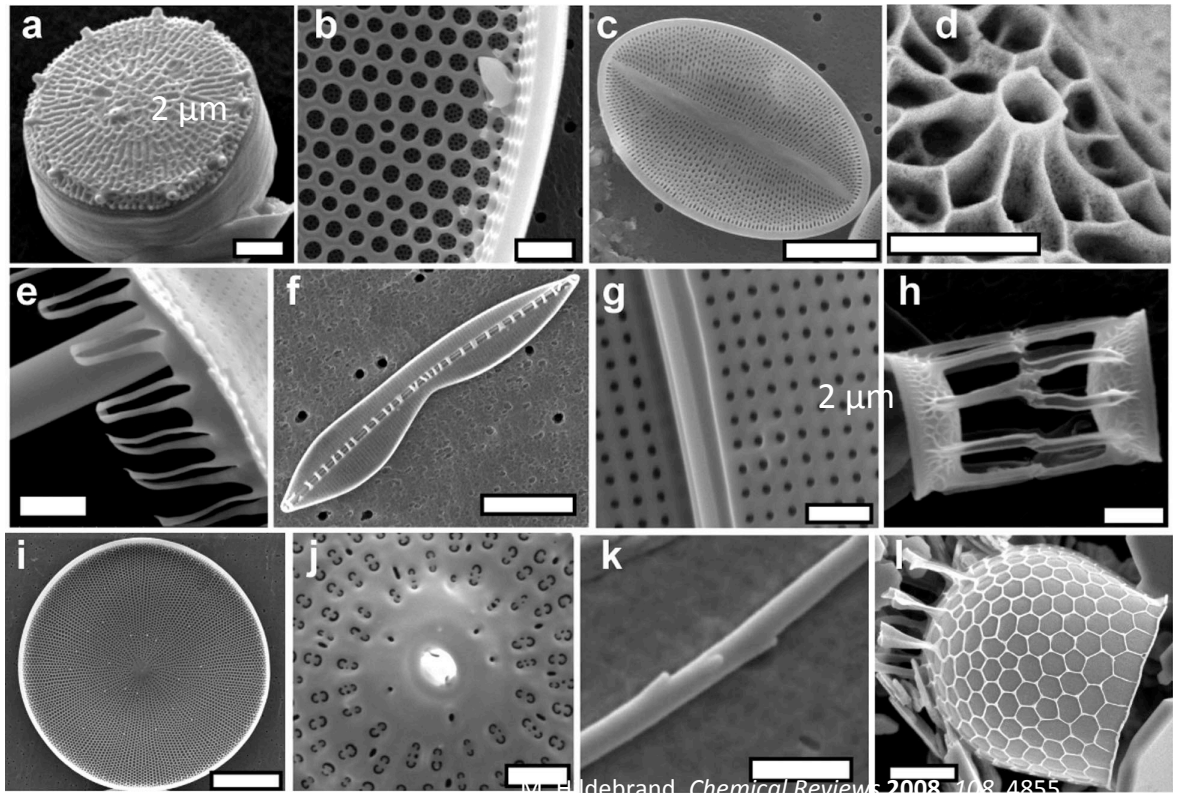
Jimin Guo, PhD UNM

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

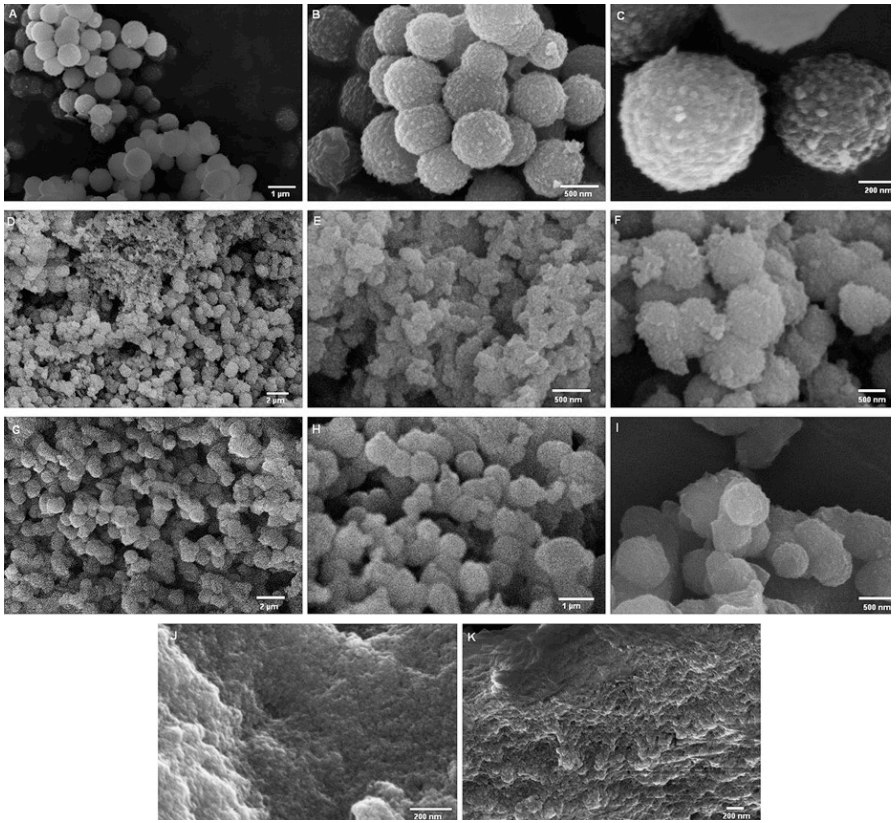


Table 1 Silica precipitating ability of various enzymes

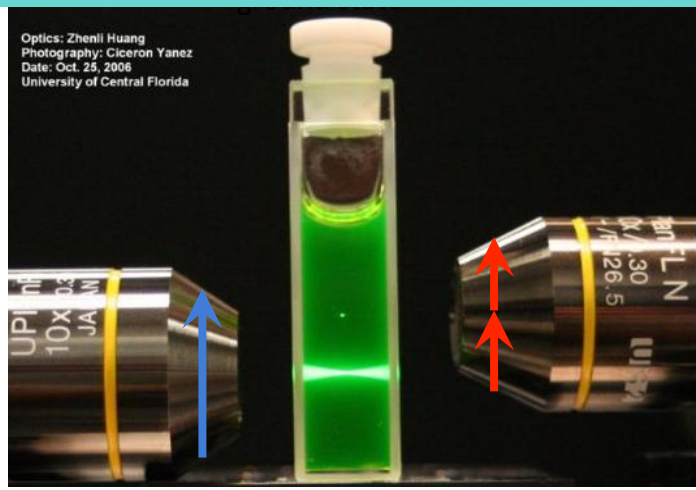
Enzyme	pI	Product	Physical state of solid silica	Time
Negative control	—	Gel		9 hours
Trypsin	10.5	Solid	Bimodal nanoparticles 100–200 nm + 700–950 nm	10 minutes
Papain	8.8–9.6	Solid	Nanoparticles 500–650 nm	15 minutes
Bromelain	9.6	Solid	Monolith	25 minutes
Tritirachium album proteinase K	8.9	Solid	Nanoparticles 450–900 nm	1 hour
Candida antarctica lipase A (CAL)	7.5	Solid	Monolith	15 minutes
Alkaline phosphatase	4.5	Gel		9 hours
Rennin	4.5	Gel		8 hours
Rhizopus oryzae lipase (ROL)	6.9	Gel		7 hours

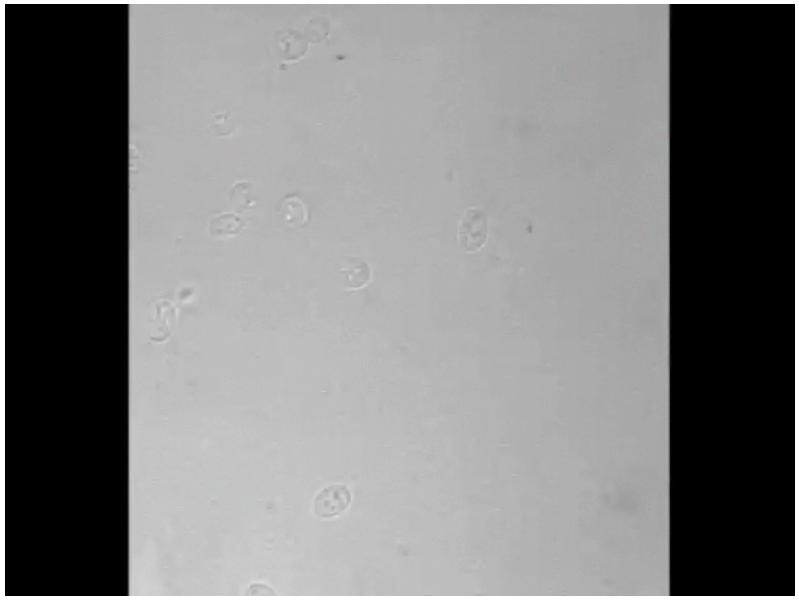
J. Mater. Chem., 2009, 19, 7606–7609

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

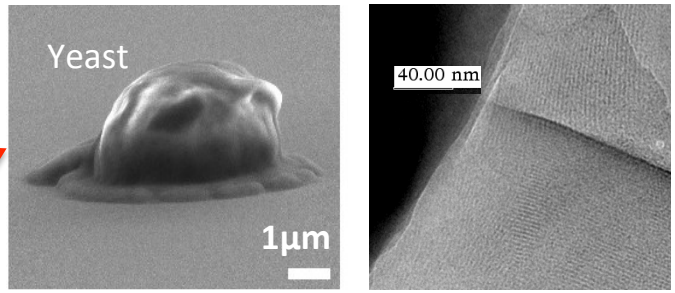


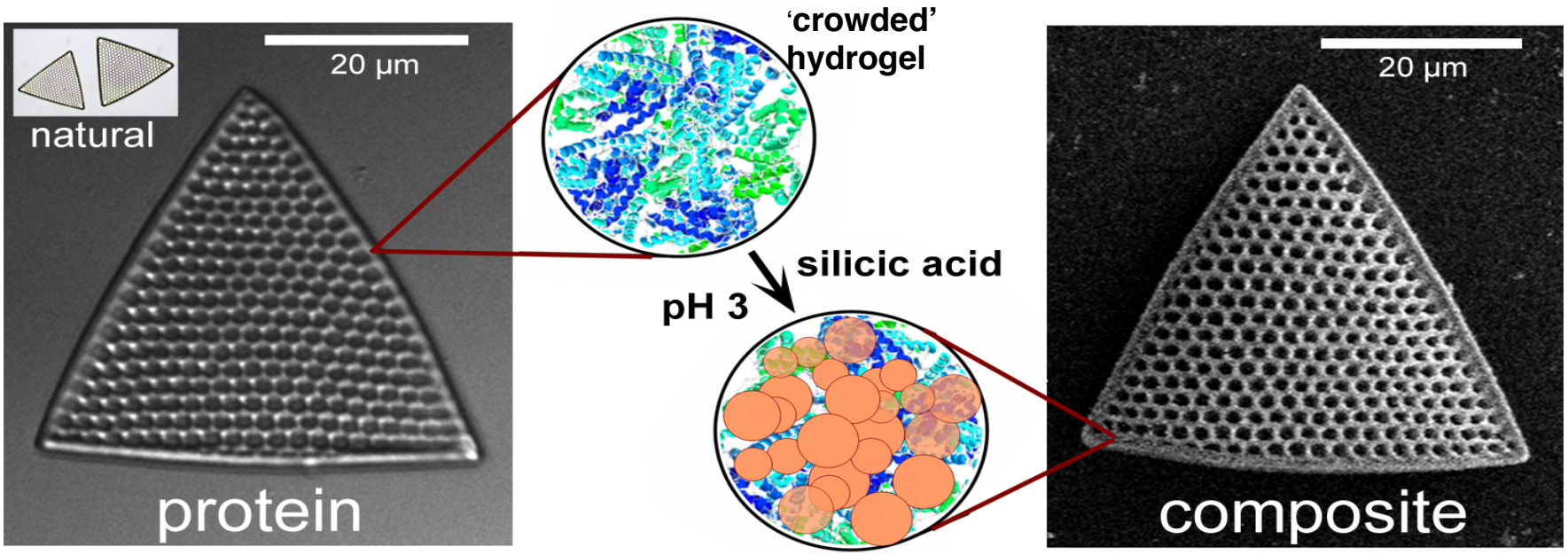


Light from a femtosecond titanium: sapphire laser is sent through a confocal scan box to raster the beam

Scaffolded MPL defined 3D protein architectures direct the formation of arbitrary user-defined silica materials

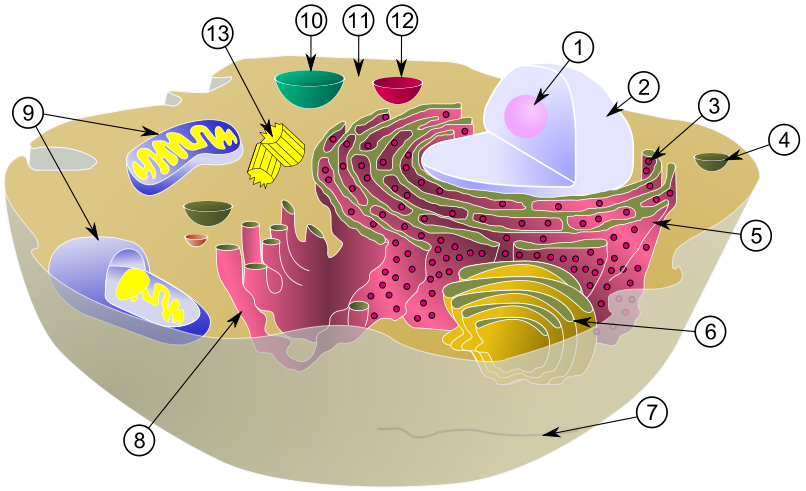
Cellular bio-molecular membrane proteins/ components may similarly direct conformal dimensionally stable silica deposition in cell-directed assembly



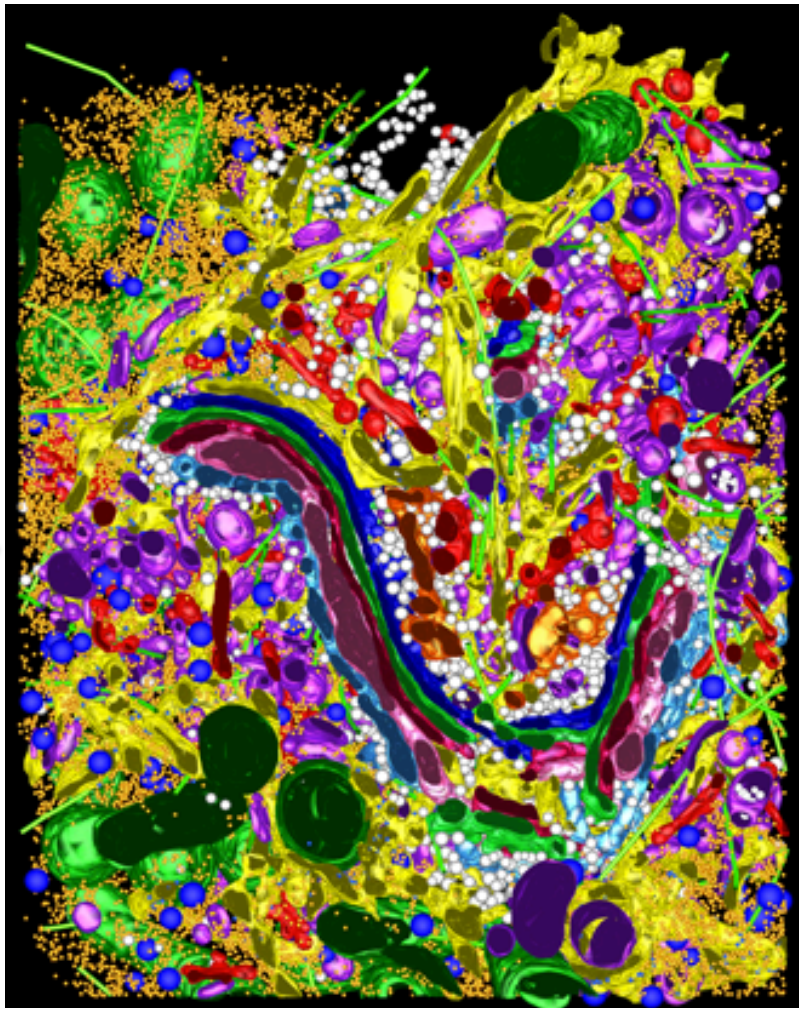


Khripin, C.Y., Pristinski, D., Dunphy, D.R., Brinker C. J., Kaehr, B.* (2011) ACS Nano, 5, 1401-1409.

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?

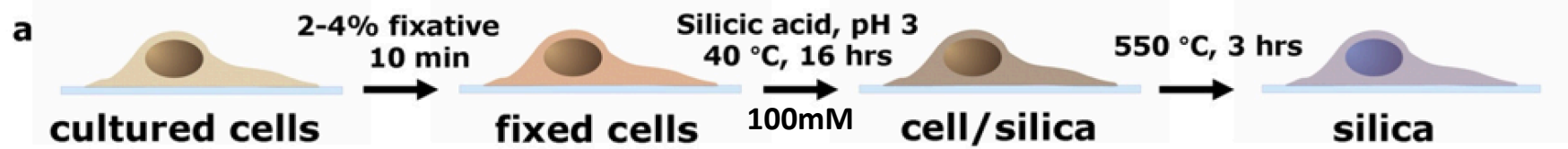


Organelles: (1) nucleolus (2) nucleus (3) ribosome (4) vesicle (5) rough endoplasmic reticulum (ER) (6) Golgi apparatus (7) Cytoskeleton (8) smooth ER (9) mitochondria (10) vacuole (11) cytoplasm (12) lysosome (13) centrioles

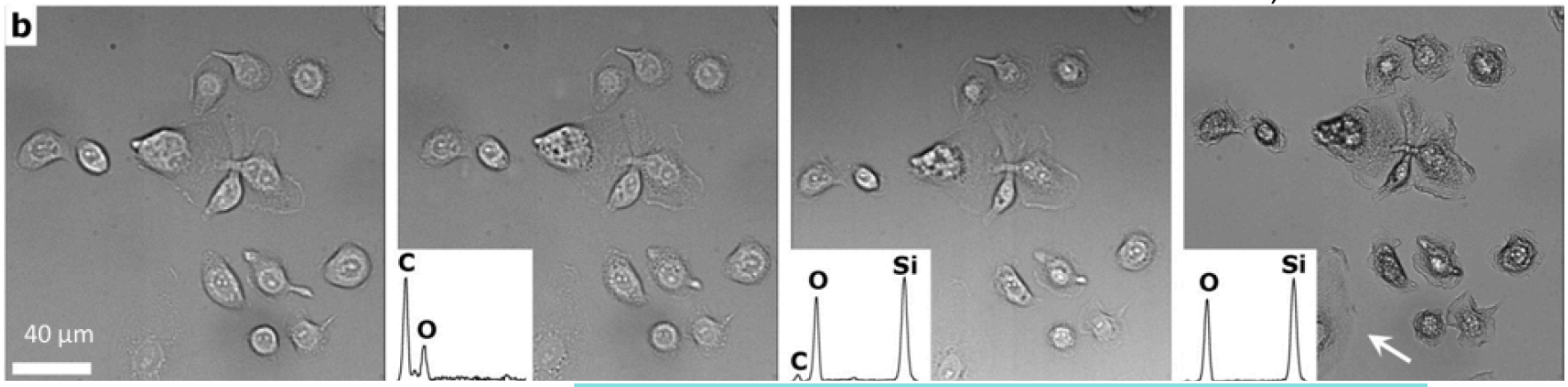


Cryo-TEM Tomography - beta cells preserved *in situ* in pancreatic islet tissue isolated from mice March et al. PNAS 2004; 101: 5565-5570

Proof: Silica Cell replication (SCR)



Kaehr, CJB et al *PNAS* 2012

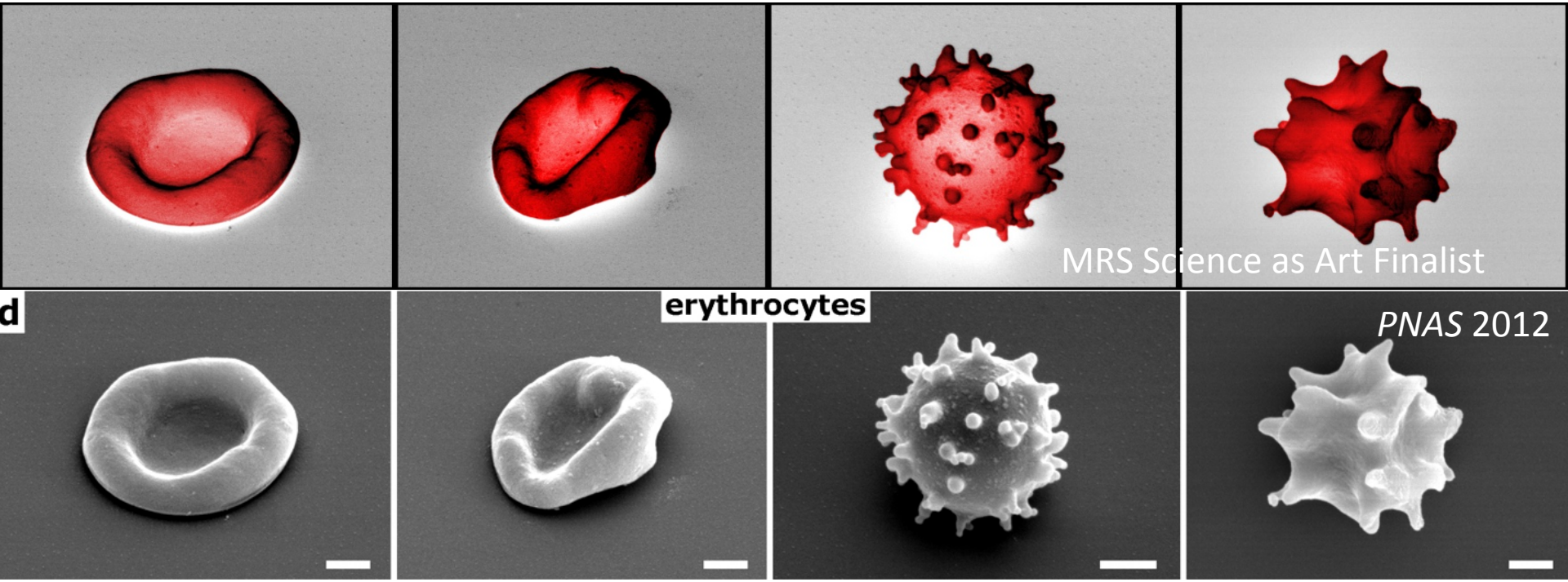


How does it work?: At ~pH 3, Monosilicic acid $Si(OH)_4$ does not self-condense. It can interchange with hydrogen-bonded interfacial water at cellular/biomolecular interfaces and be concentrated and catalyzed amphoterically by proximal membrane associated and globular proteins (and perhaps other components)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

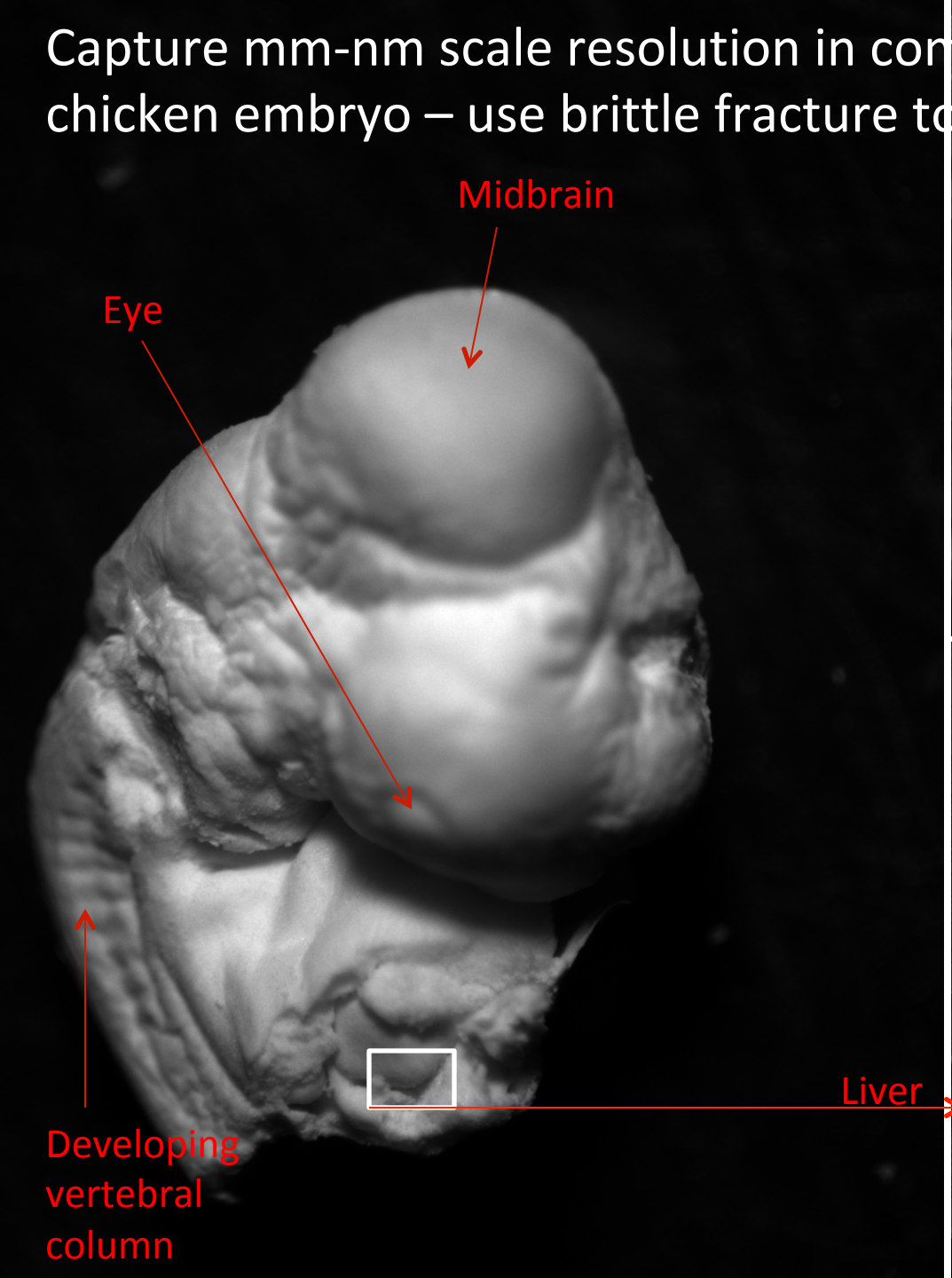
Blood cells and their varying morphologies induced by osmotic stress are replicated with high fidelity

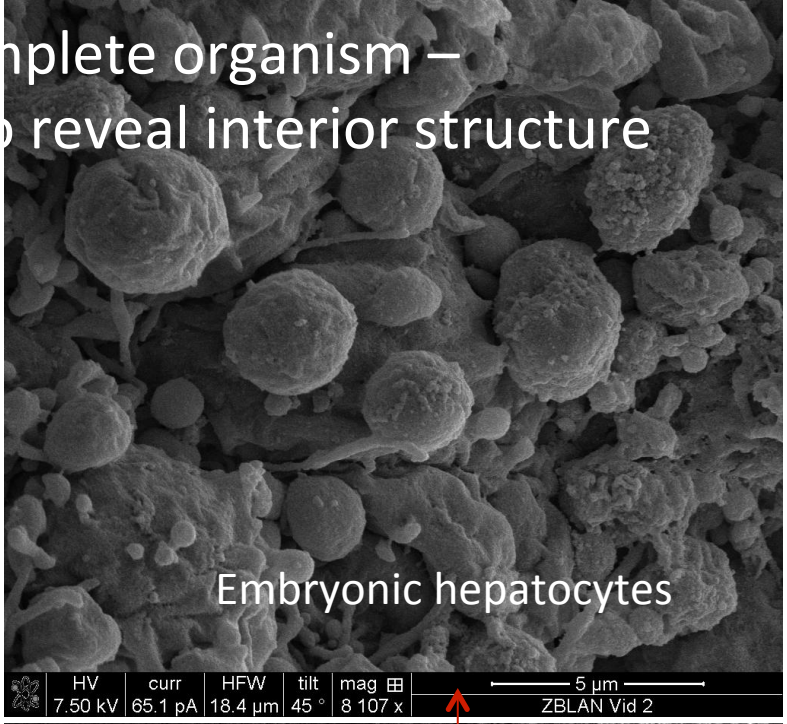


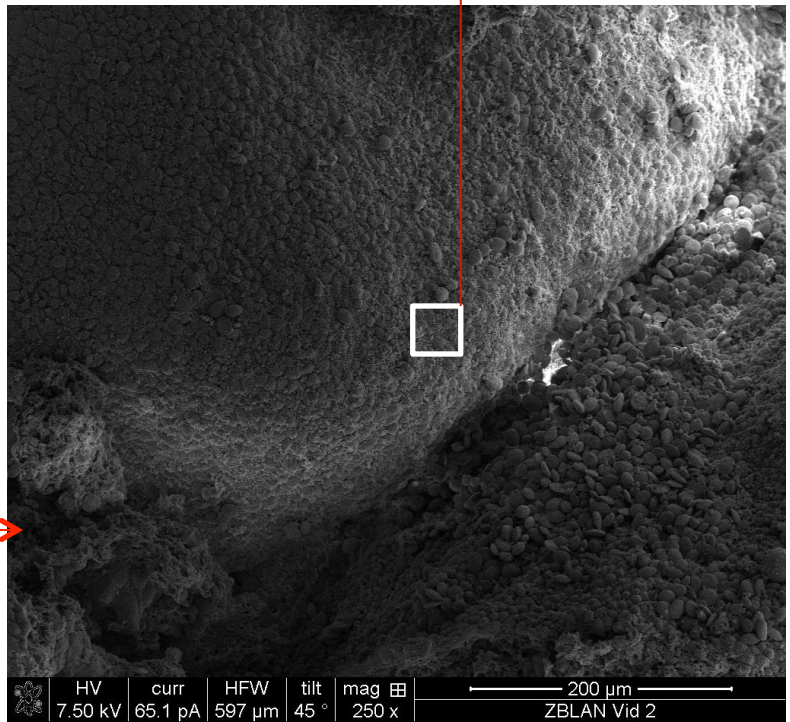
Scale bars = 1µm

- 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

Capture mm-nm scale resolution in complete organism – chicken embryo – use brittle fracture to reveal interior structure

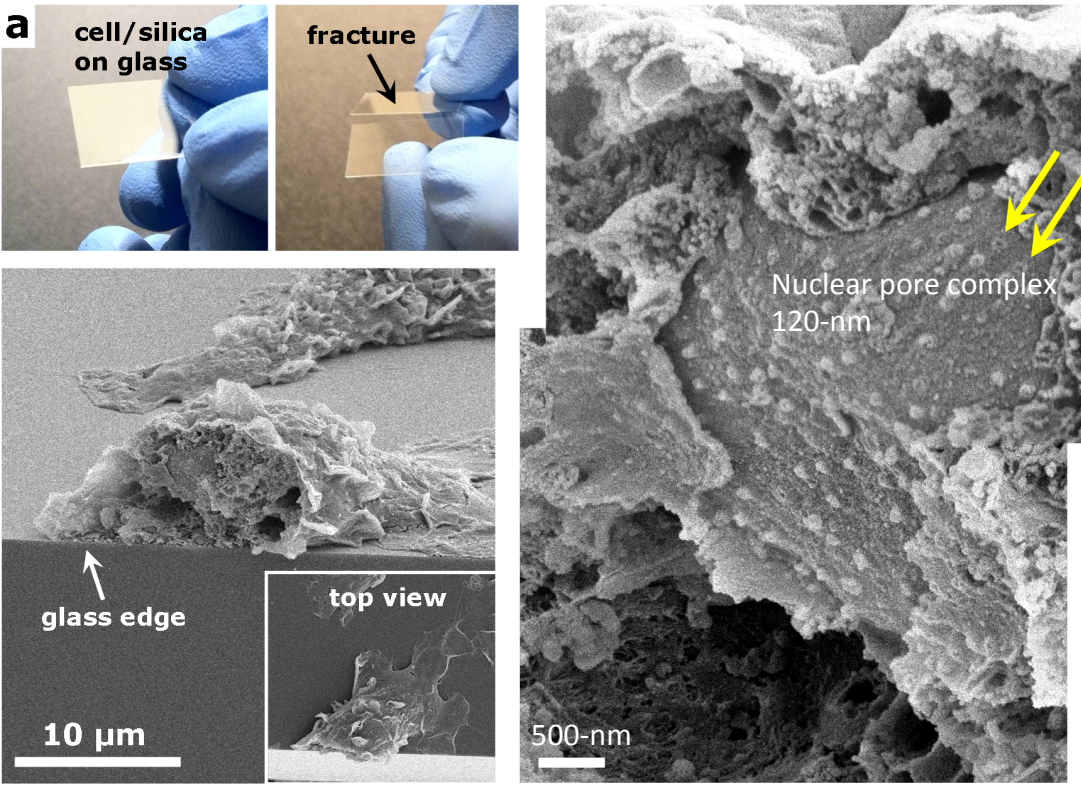


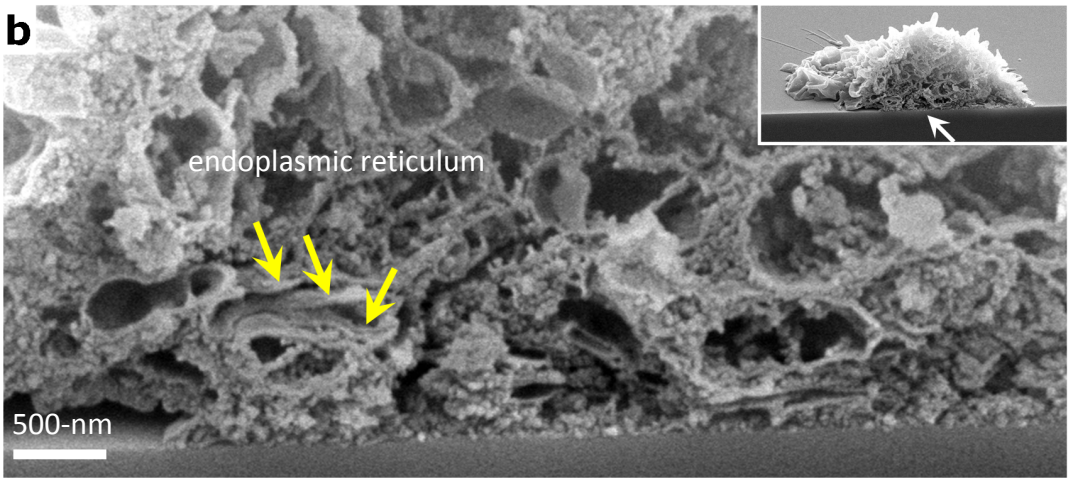


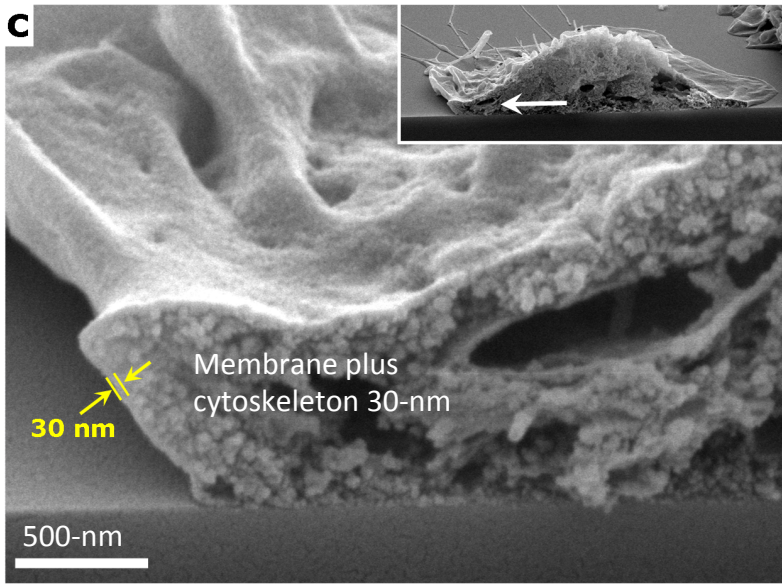


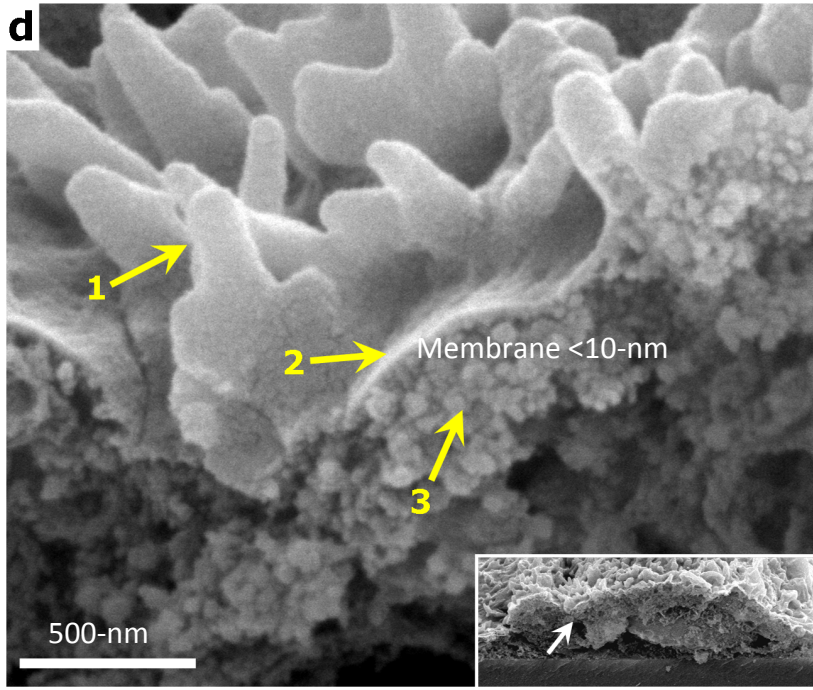
‘Brittle’ Fracture of the Organism: Allows us to capture with high fidelity selected regions within the interior the organism – here red and white blood cells within the venous system of the chick embryo liver

What is the ultimate resolution/precision of the replication process?

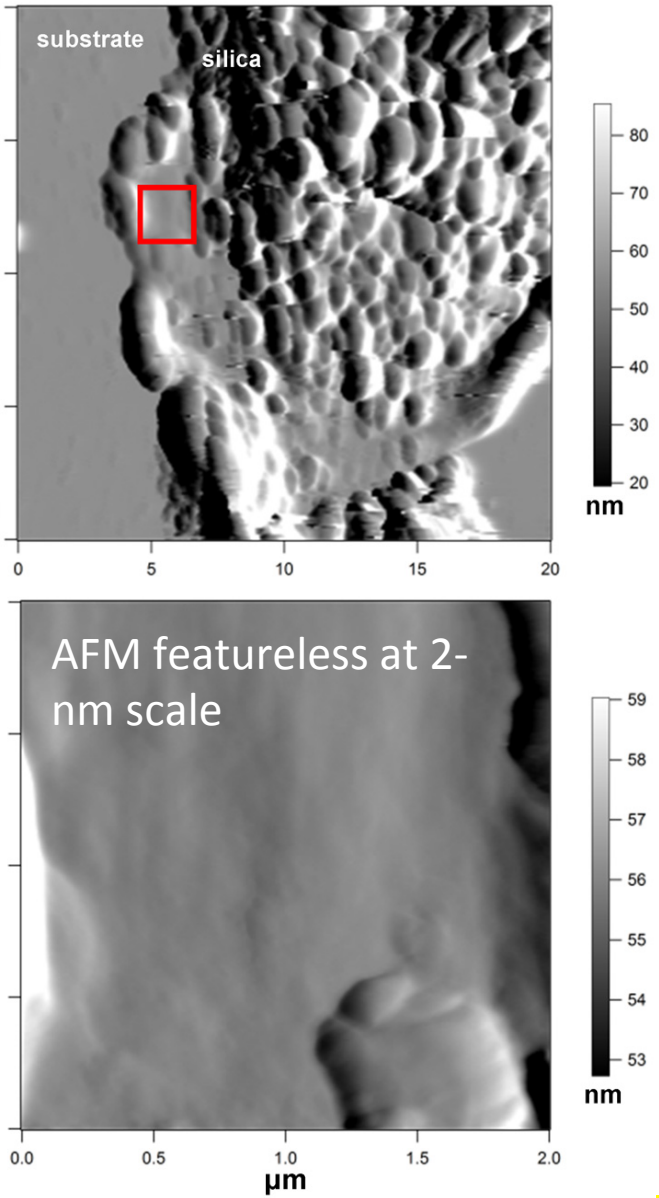


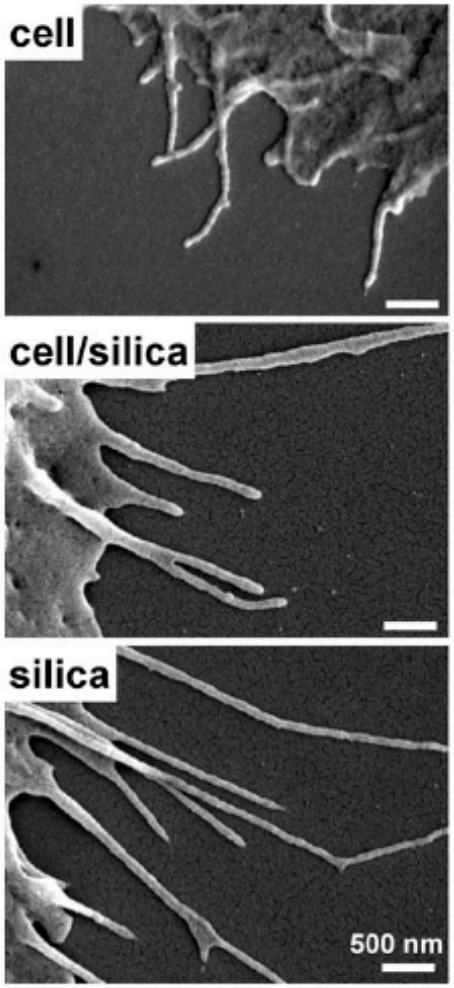


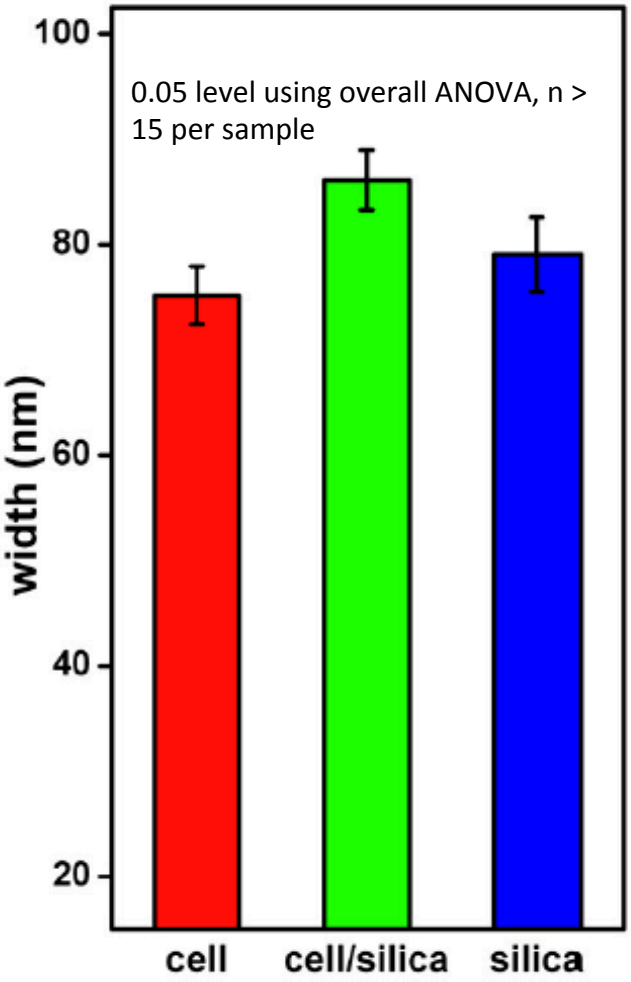




What is the ultimate resolution/precision of the replication process?



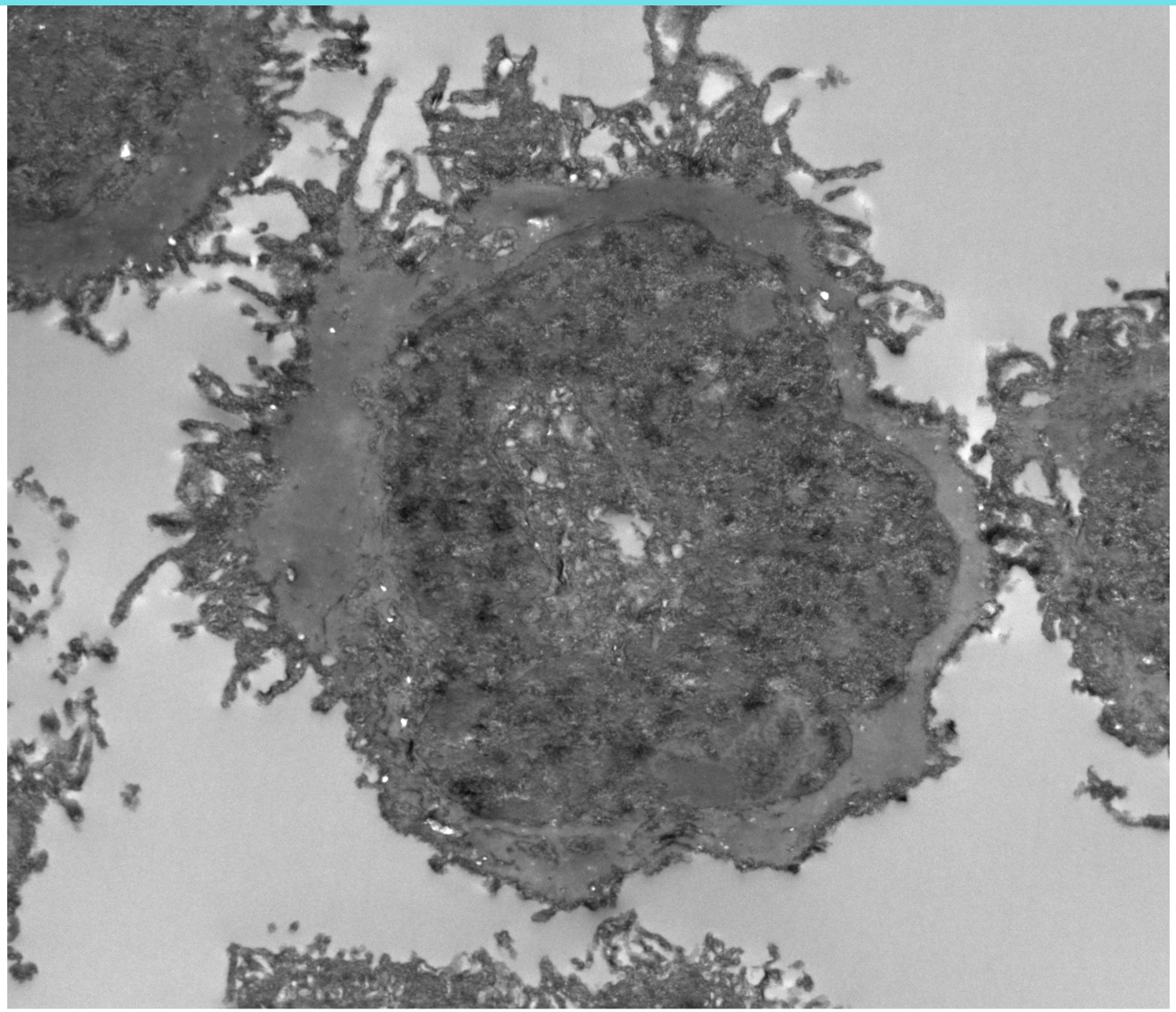




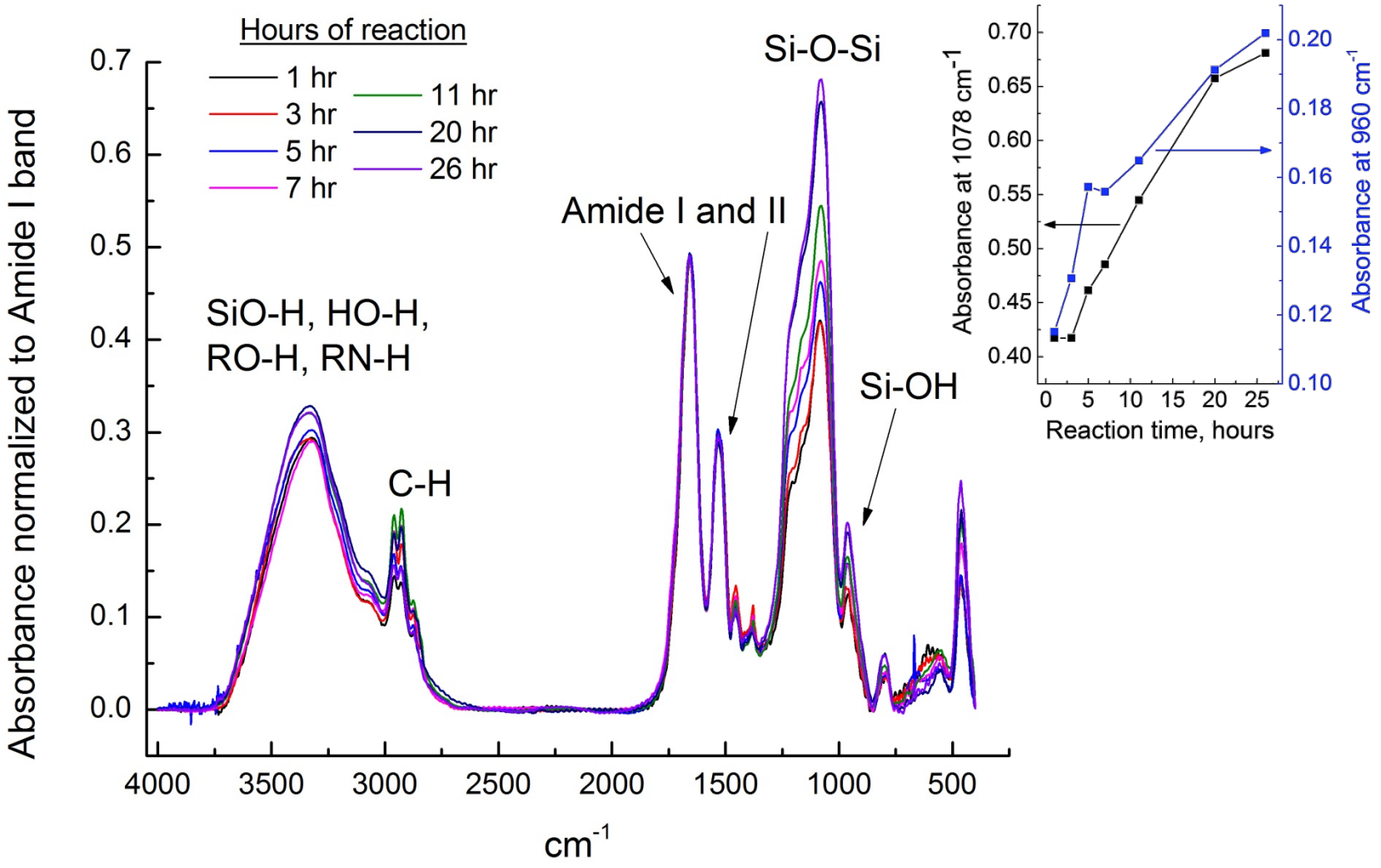
Feature dimension suggests silica deposition thickness limited to < 6-nm; calcination results in cell replica with ~2-nm precision, AFM featureless at 2-nm scale

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

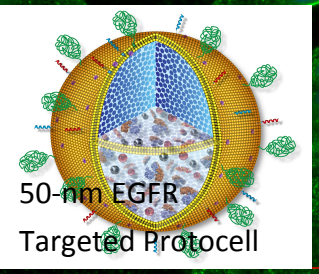
Conventional Microtome preparation shows morphological details nearly indistinguishable from the parent cells



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



50-nm EGFR Targeted Protocell

A549, 0 h

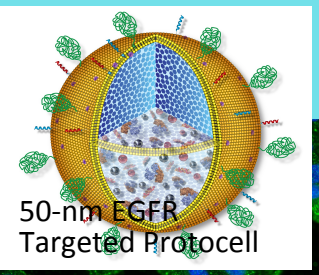
A549, 2 h

A549, 4.5 h

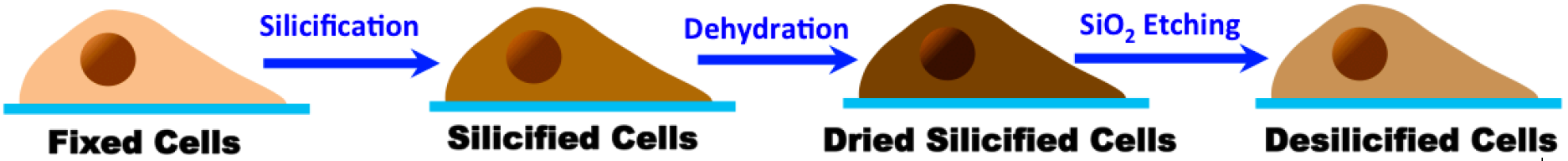
A549, 6 h

20 µm

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



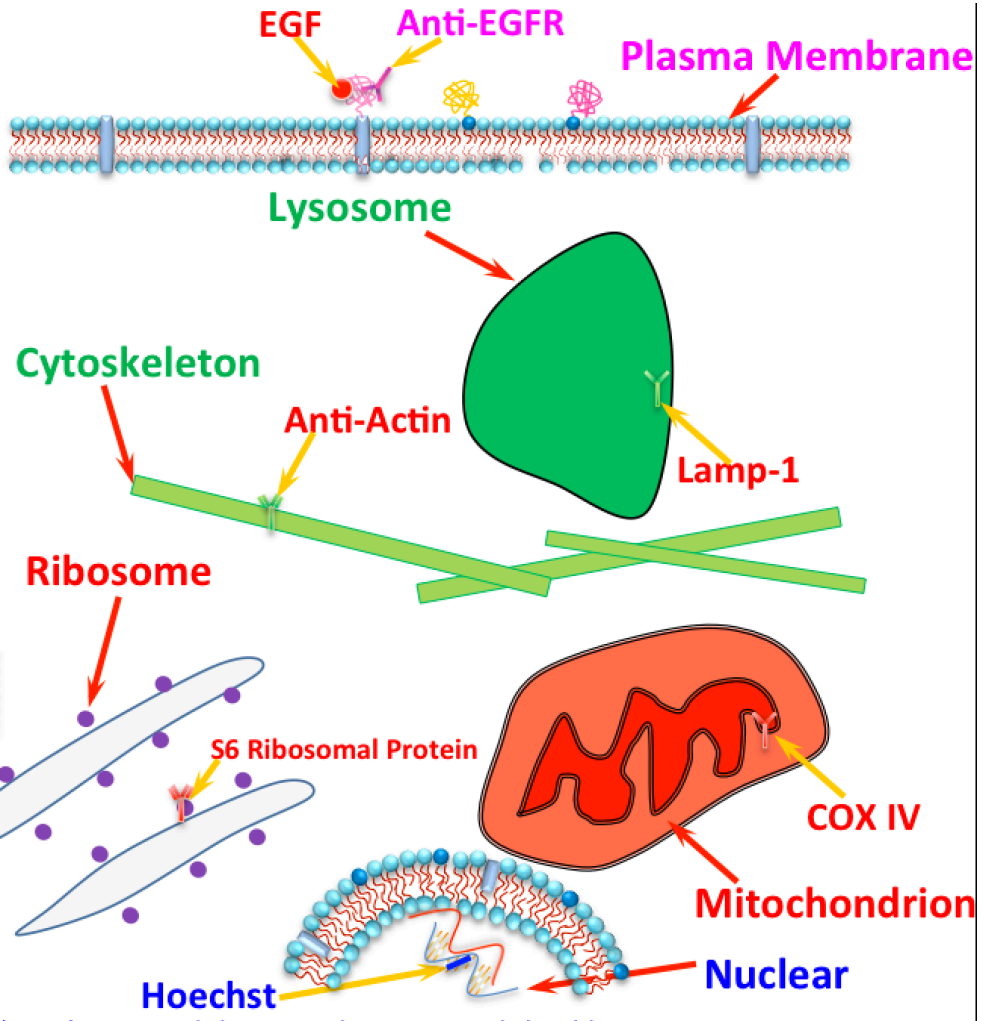
We probed the accessibility and biofunctionality of cell surface and intracellular antigens, receptors, and bio-markers with molecular dyes, ligands, and antibodies with different molecular weights and characteristic dimensions



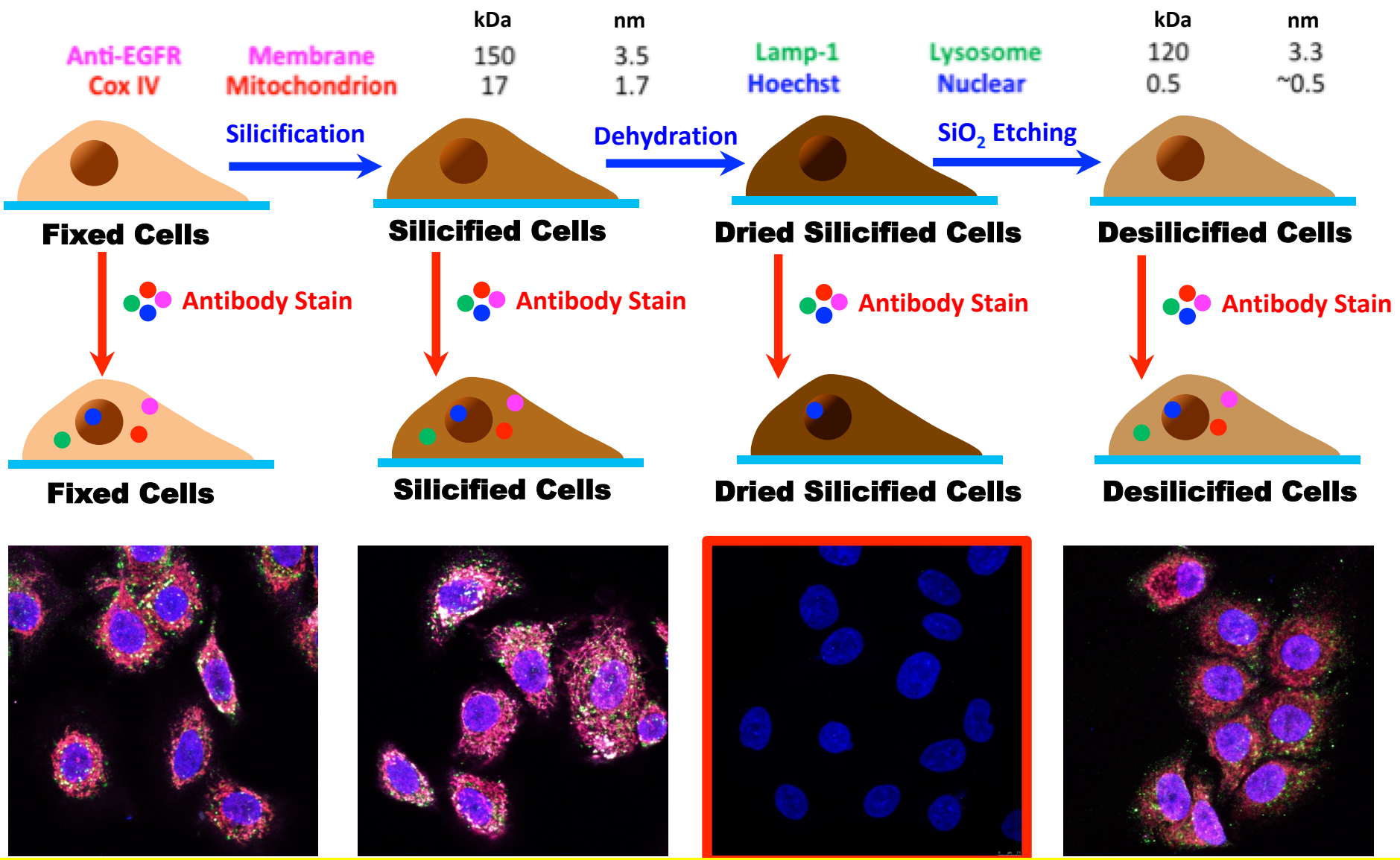
Biomarker	Position on/in the cell	Approx. Size, M (kDa)	Radius R_{min} (nm)
Anti-EGFR	Membrane	150	3.5
EGF	Membrane	6	1.2
Lamp-1	Lysosome	120	3.3
Cox IV	Mitochondrion	17	1.7
S6 Ribosomal Protein	Ribosome	32	2.1
Anti-Actin	Cytoskeleton	43	2.3
Hoechst	Nuclear	0.5	~0.5
EGFR targeted NP	Membrane		50

$R_{min} = 0.066M^{1/3}$

(for M in Dalton; R_{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) – 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

Jimin Guo

Biomarker	Position on/in the cell	Approx. Size, M (kDa)	Radius R_{min} (nm)
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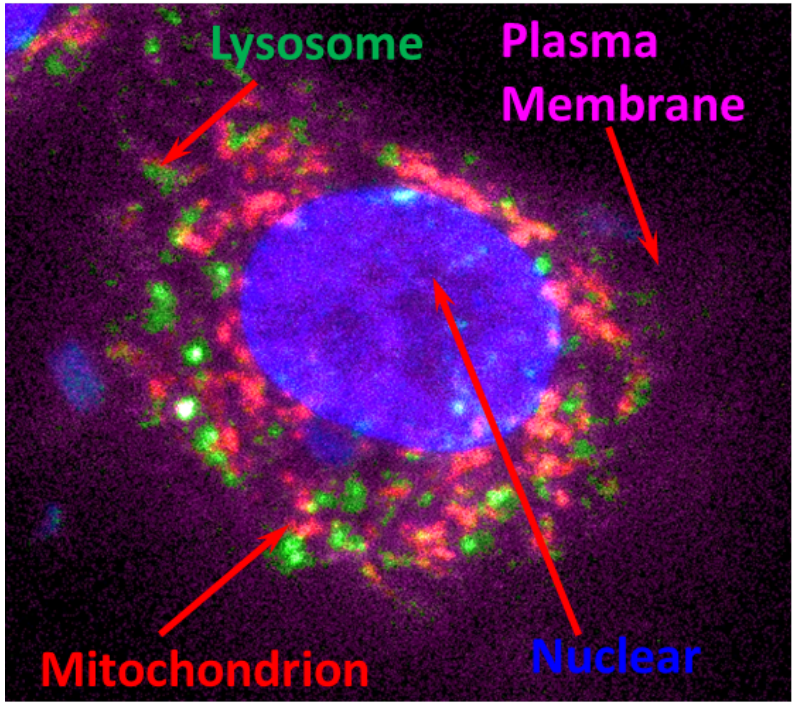
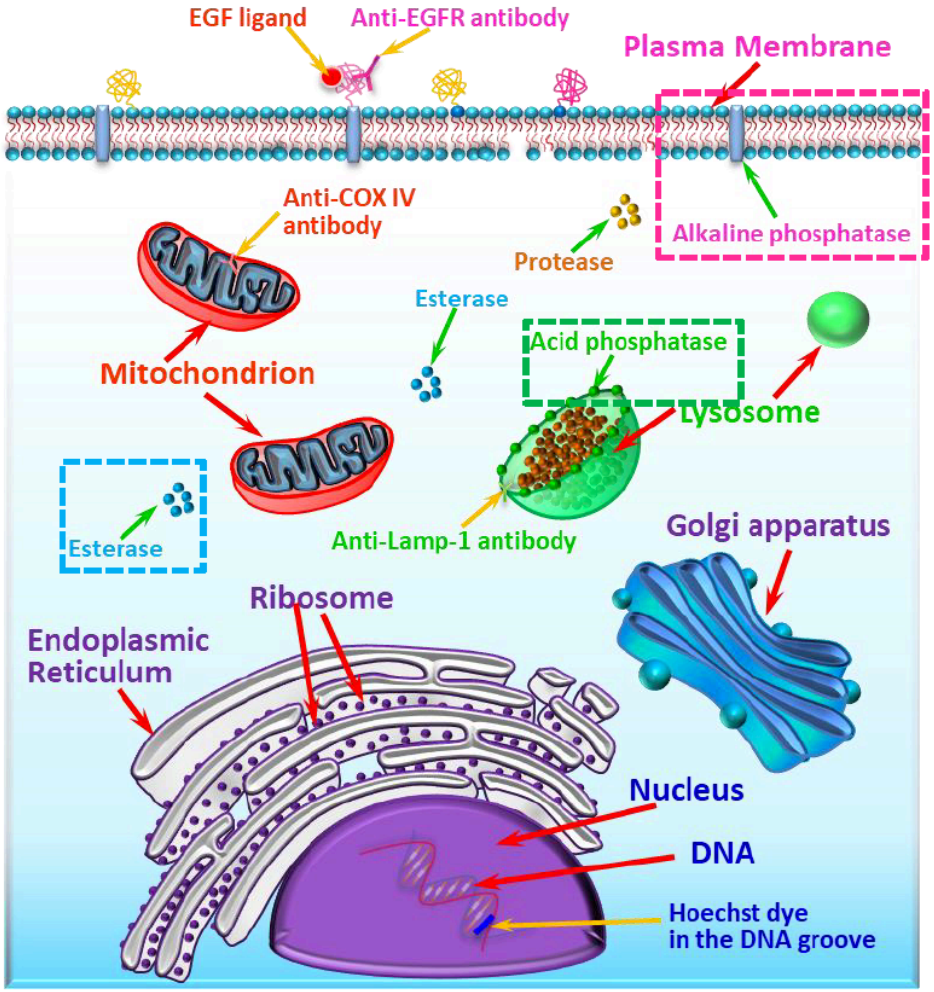
S6 Ribosomal Protein	Ribosome	32	2.1
Anti-Actin	Cytoskeleton	43	2.3
Hoechst	Nuclear	0.5	~0.5
EGFR targeted NP	Membrane		50

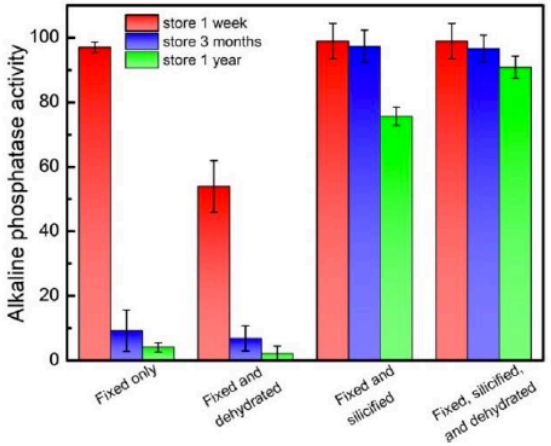


A549 Cell following Silicification, Drying, and SiO_2 Etching

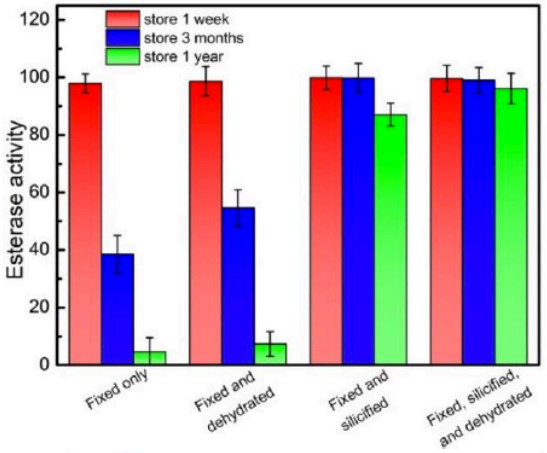
We observe remarkable fidelity of preservation of dimensions and biomolecular recognition upon silicification, drying, and etching: stable vaccines, cellular decoys…

Long Term Preservation of Enzyme Activity

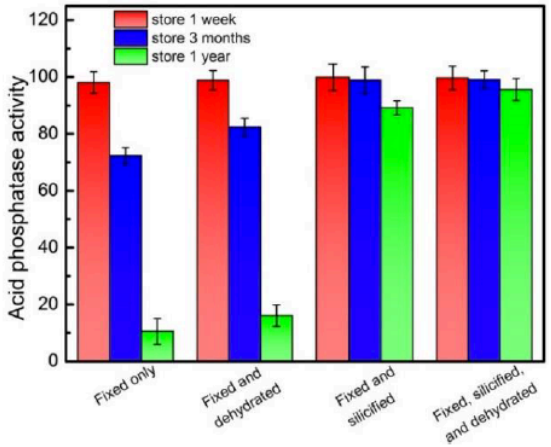




Alkaline phosphatase



Esterase

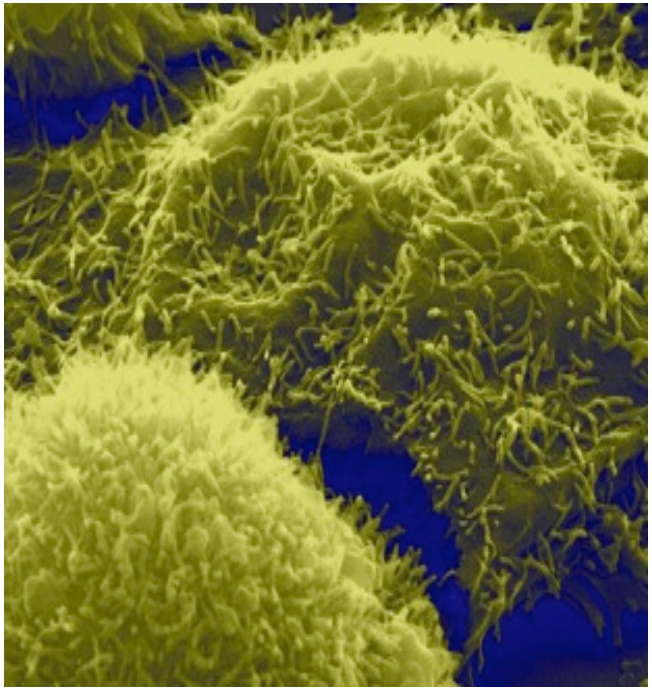


Acid phosphatase

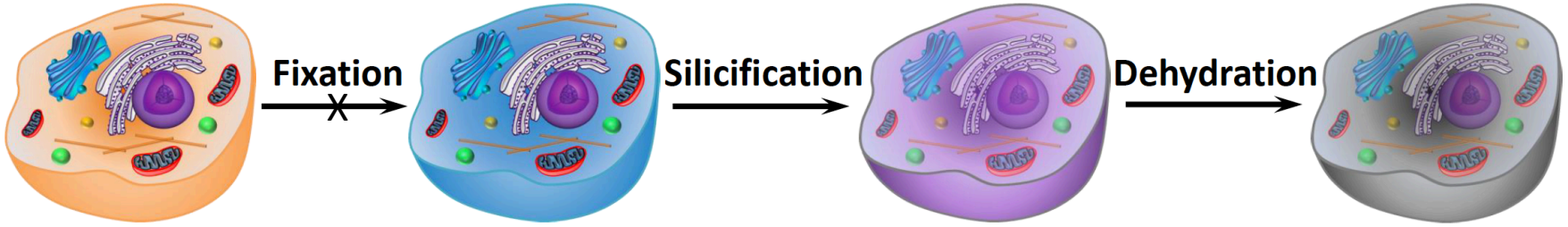
Dehydration seals the system (surface area disappears) and prevents degradation

Biomineralized Cancer Cell Vaccines – produced from replicas w/o fixation

- If $Si(OH)_4$ and H_2O 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?



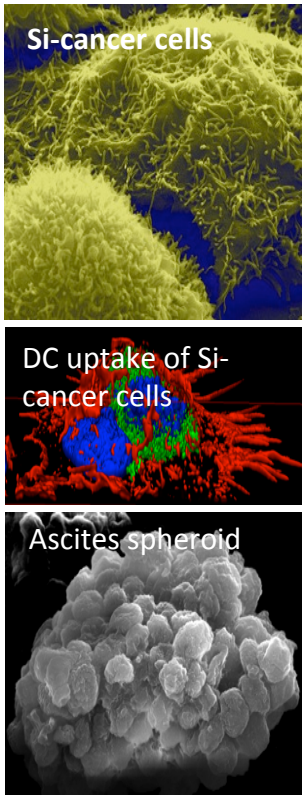
Silica Cell Replication



Rita Serda, Jimin Guo et al Patent Pending

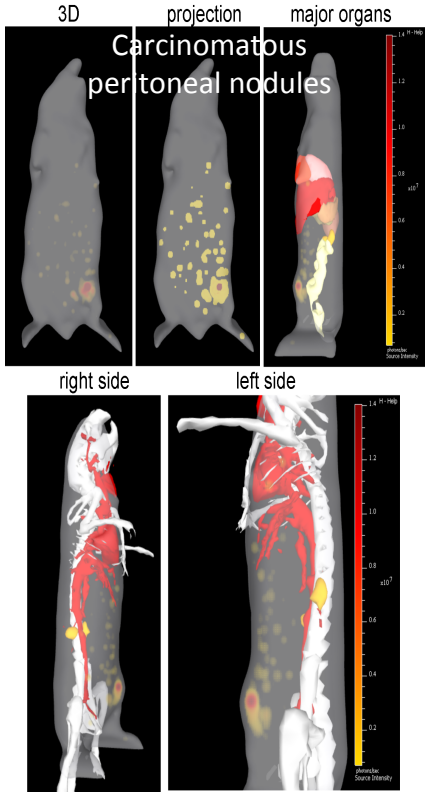
Biomineralized Cancer Cell Vaccines

Silicified BR5-Akt ovarian cancer cells modified with surface PAMPs



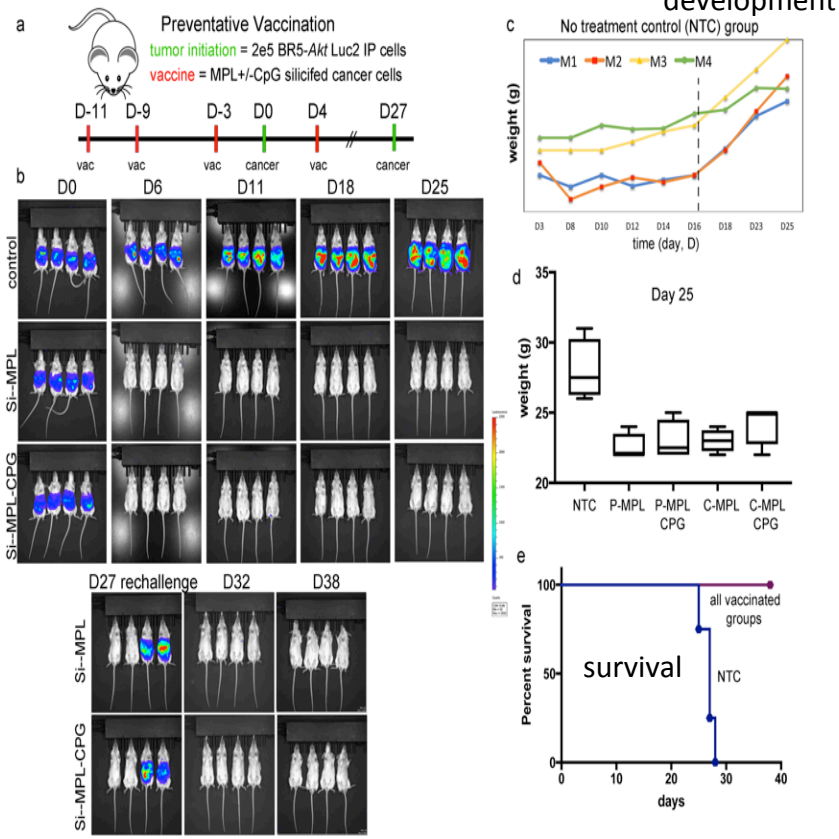
IP

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



Prevention of tumor engraftment

ascites development



Luciferase-expressing ovarian cancer cells

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

UNM Comprehensive Cancer Center

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

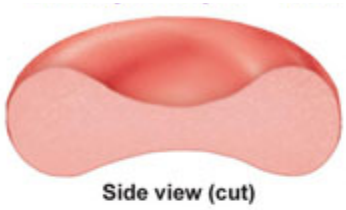
- Ultimate nanostructure can be featureless and defect-free ~ 2-nm precision

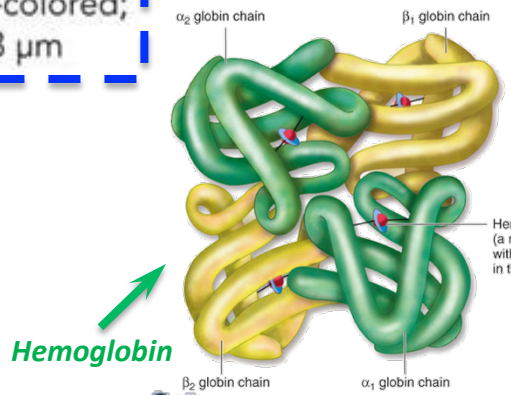
- Preserved biomolecular structure (FTIR) and functionality (enzymatic activity and antigen presentation)
  - Dried replica can be stored and re-activated with water or etching
  - Enzymatic activity preserved for >1 year in dried samples
  - Can we avoid/reverse fixation?

- **Cancer Vaccine Demonstrated**

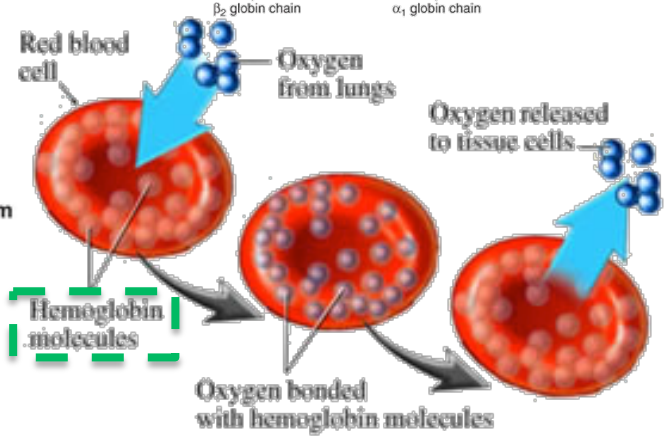
Red blood cells (RBCs) possess many unique characteristics, including ***special shape, flexibility***, ***the ability to carry oxygen***, and ***long circulation times*** – **can we create stable, synthetic, long-circulating analogues?**

Illustration	Description*	Number of Cell per mm^3 (μl) of Blood	Function	Duration of Development (D) and Life Span (LS)
	Biconcave, anucleate disc; salmon-colored; diameter 7–8 μm	4–6 million	Transport oxygen and carbon dioxide	D: 5–9 days LS: 100–120 days

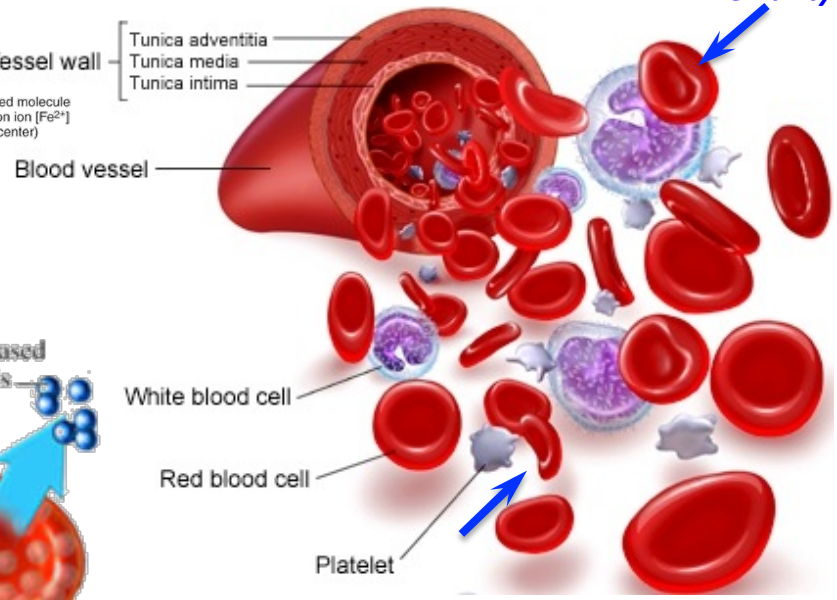




Hemoglobin



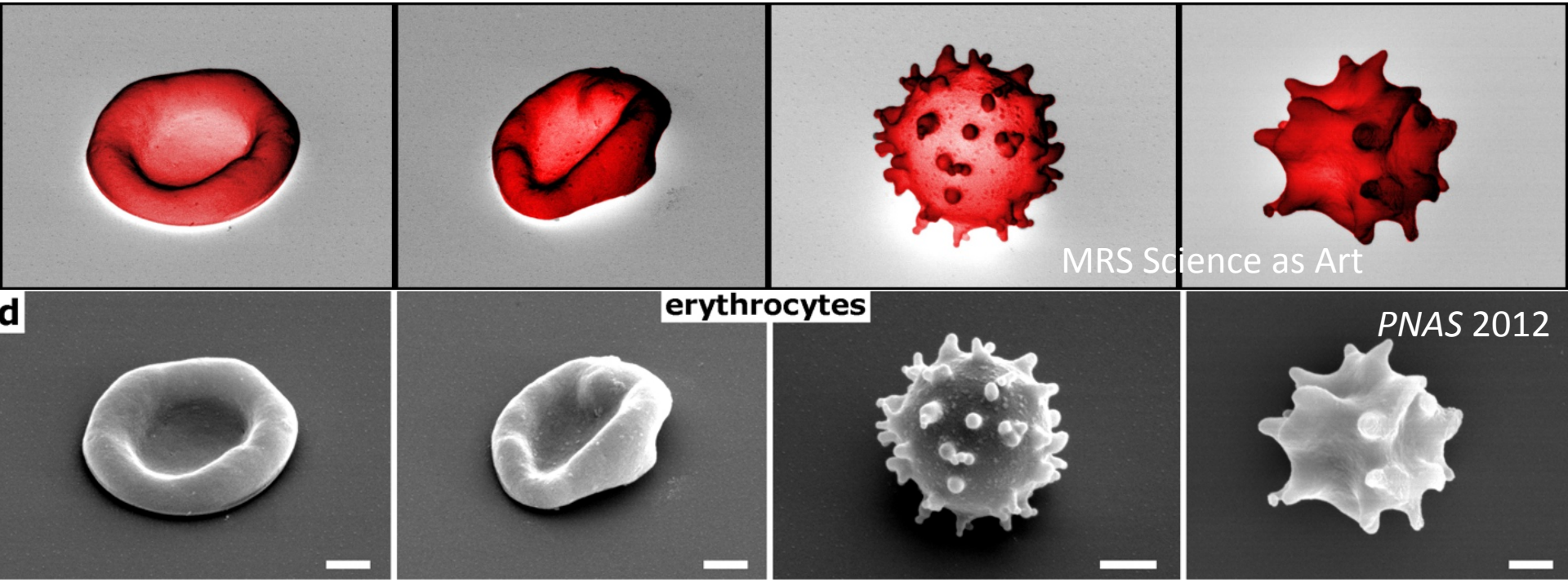
Flexibility



Antranik. "Blood Components, Hemoglobin, Type/Rh Factor, Agglutination." Antranik (2011) Dec. 4. http://www.majordifferences.com/2013/03/difference-between-rbc-and-wbc.html#.WBUOwDUYOhg

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



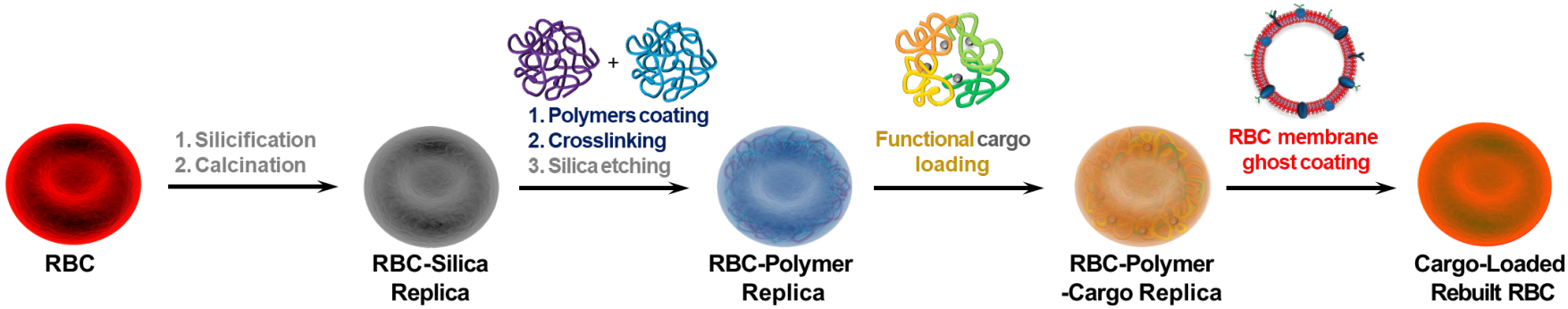
Scale bars = 1µm

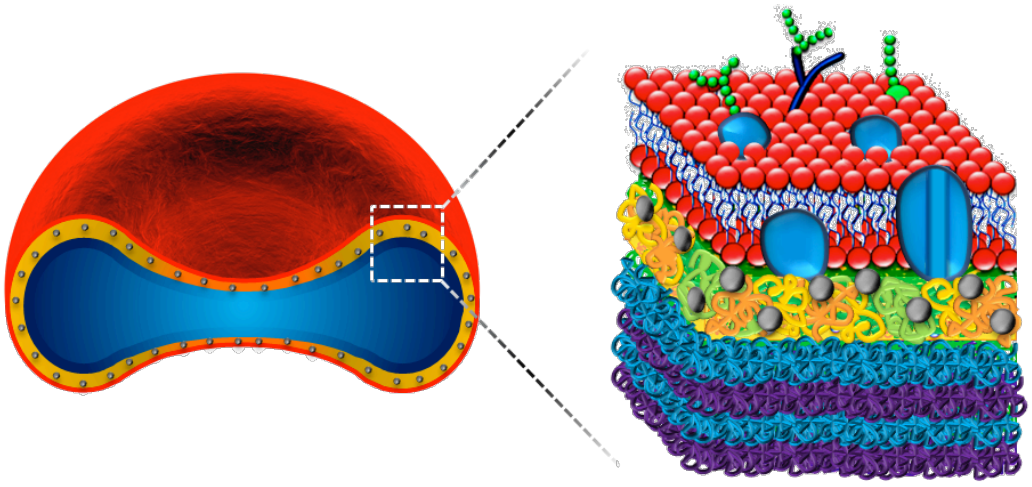
•Increasingly abnormal/crenate morphology resulting from increasing levels of osmotic stress – energy consumed/transduced to alter cell shape and protein expression, which are protected and preserved within silica and transformable to other chemistries

• Cells can be decorated with NPs etc. prior to shape change

Hypothesis and Experimental Design

Silica cell replication combined with layer-by-layer assembly and native RBC membrane fusion enables construction of a multifunctional artificial RBC platform.





MEMBRANE

Self-antigens & Immune-evasive

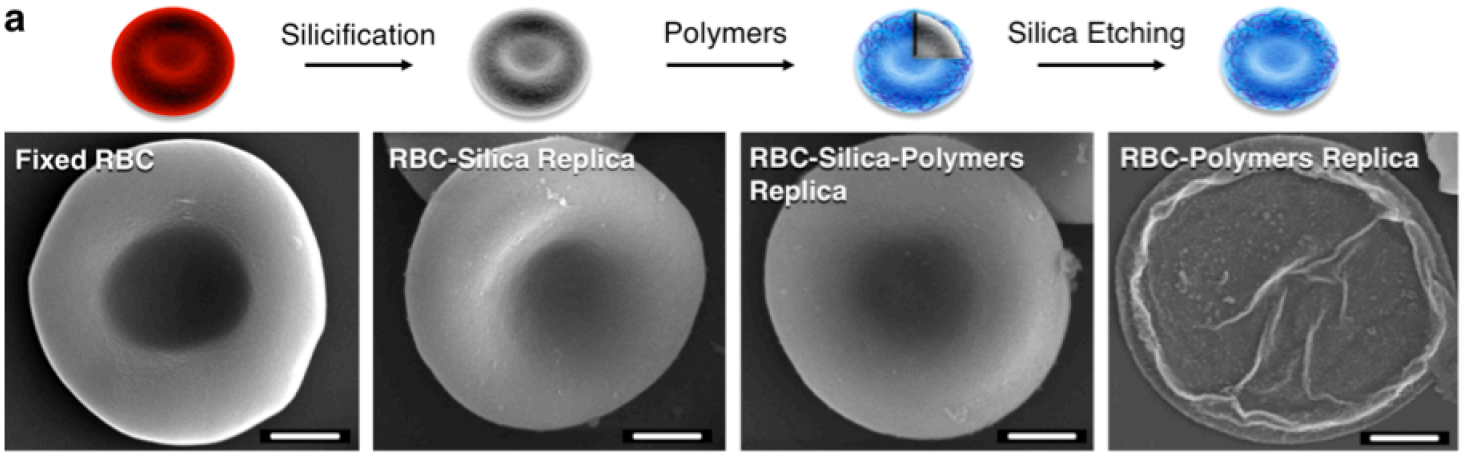
FUNCTIONAL CARGO

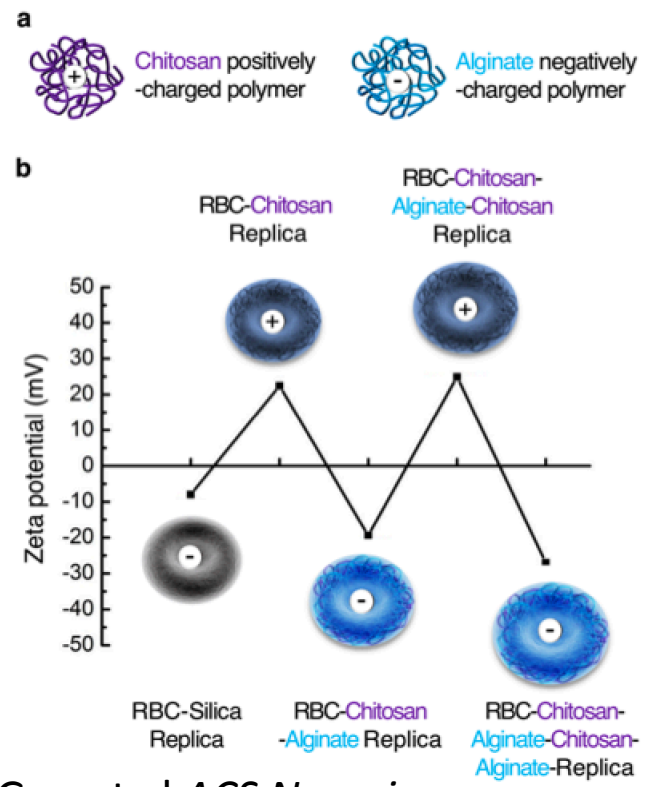
Cargo delivery & Bio-detection

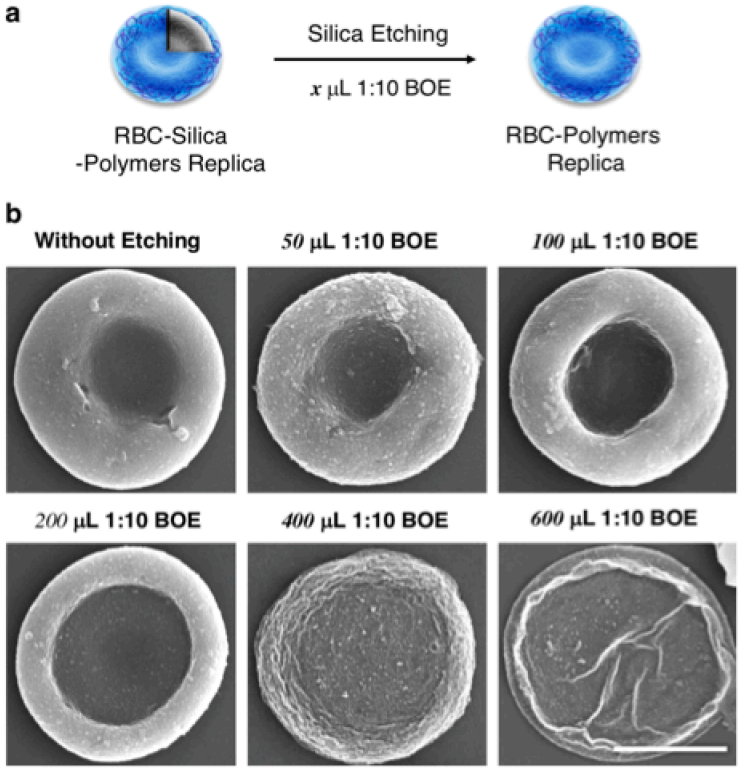
POLYMER CORE

Biconcave shape & Deformability

Fabrication of Rebuilt red blood cells (RRBCs)







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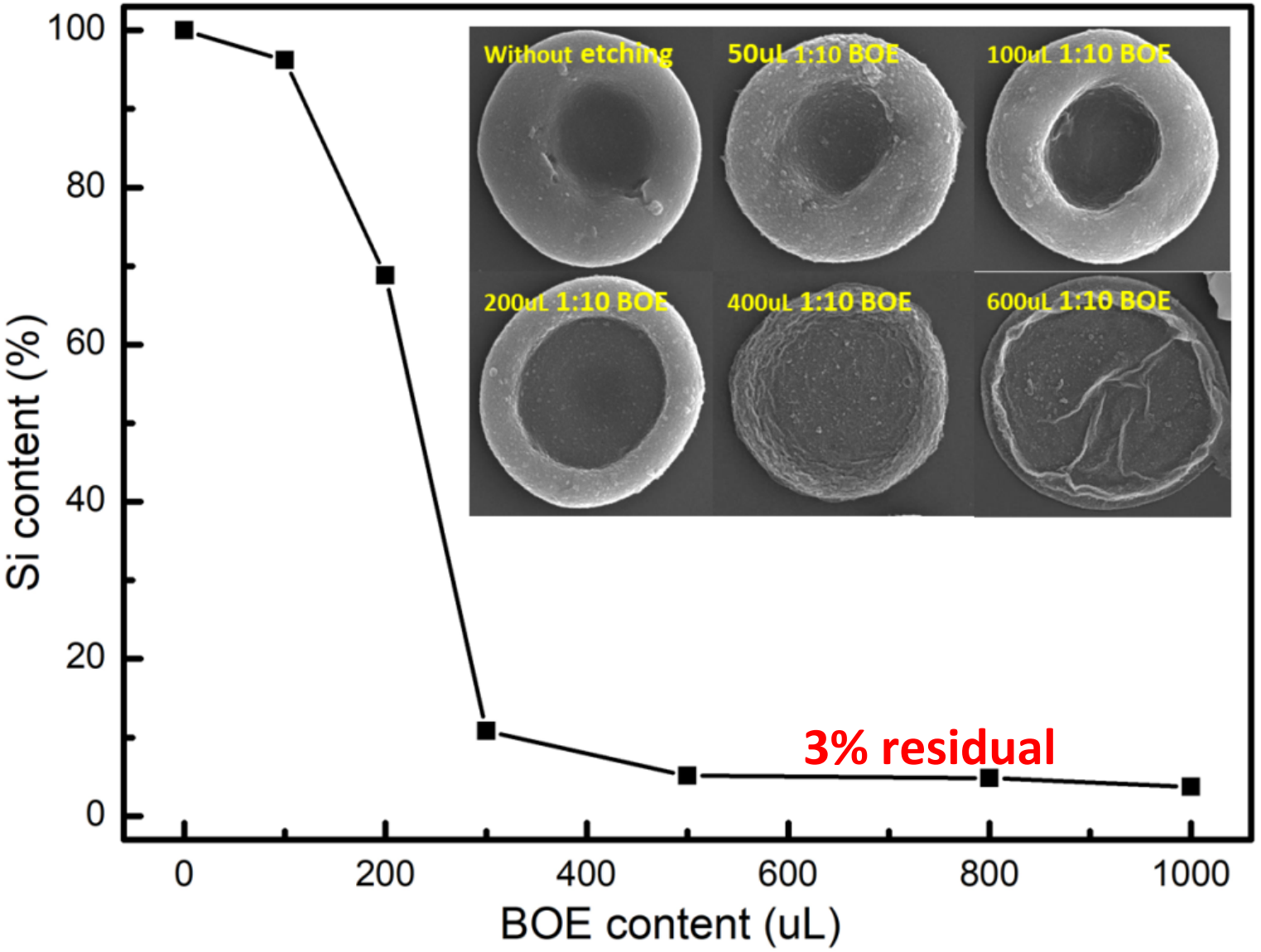
Buffered Etching Process – Inside-out precision etching of silica – residual silica at polymer/replica interface preserves RBC shape



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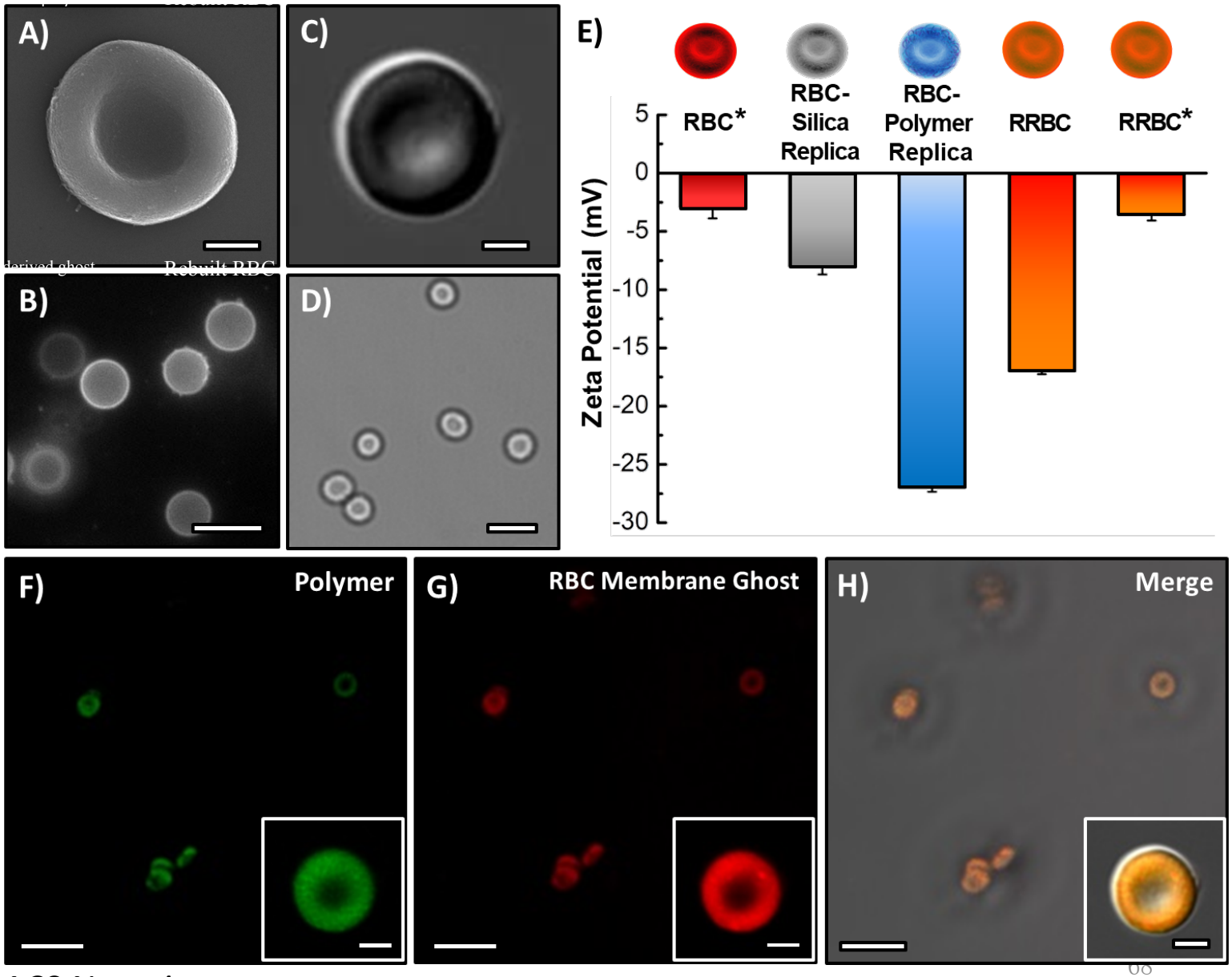
RRBCs maintain identical biconcave shape and charge as native RBCs



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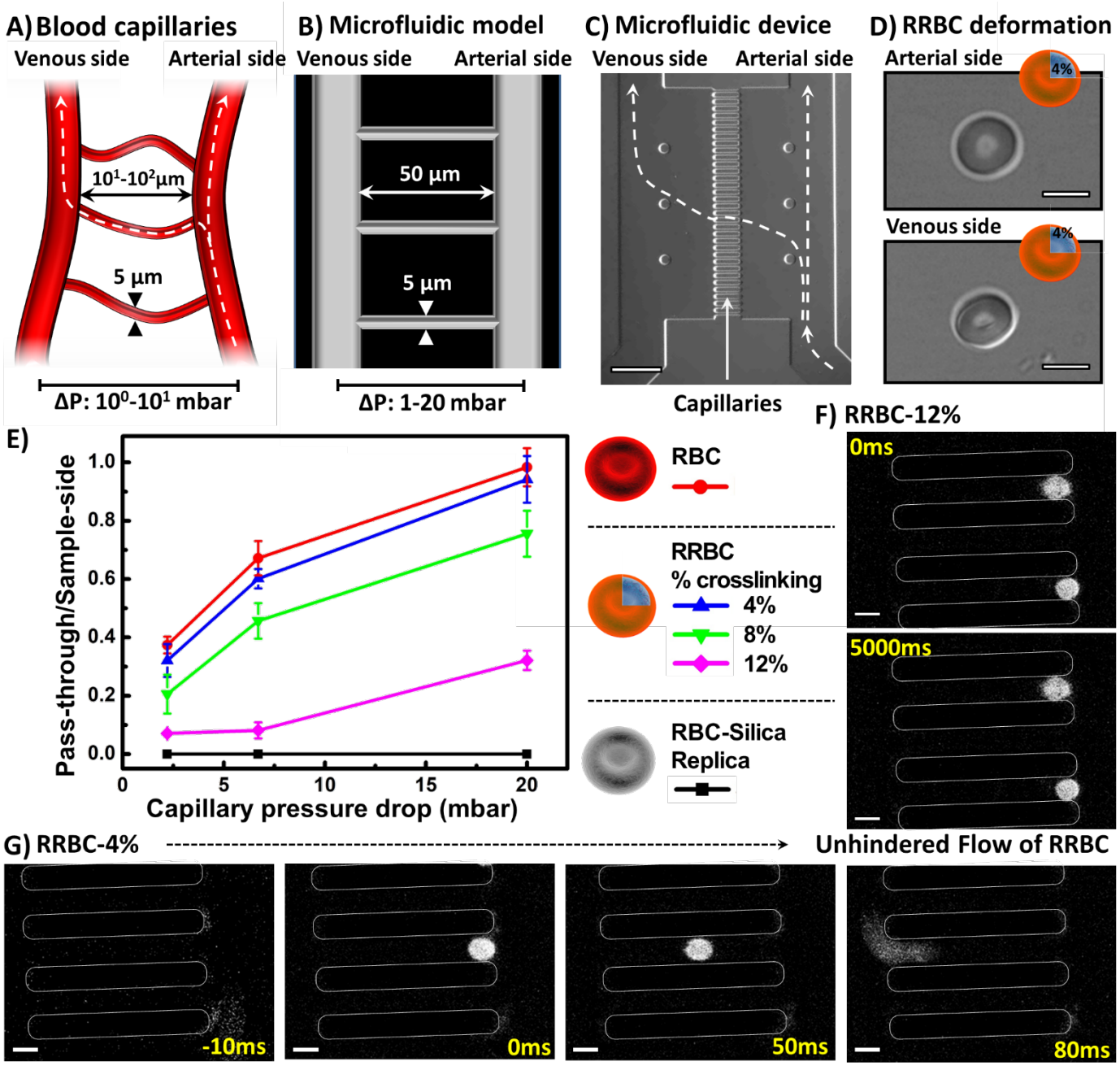
RRBCs deform and reconstitute their shape like native RBCs



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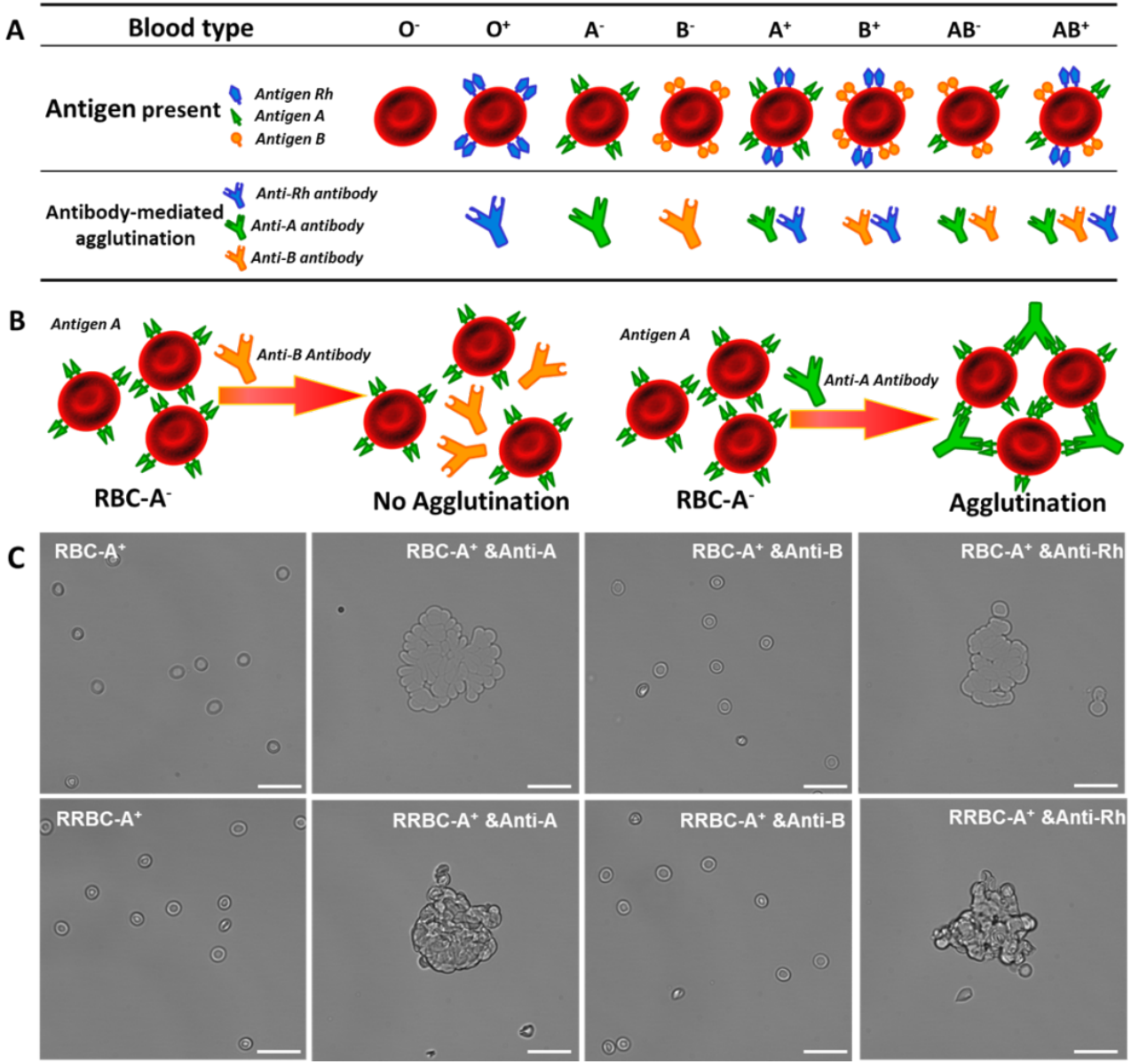
Surface Self-Antigens *are preserved RRBCs*



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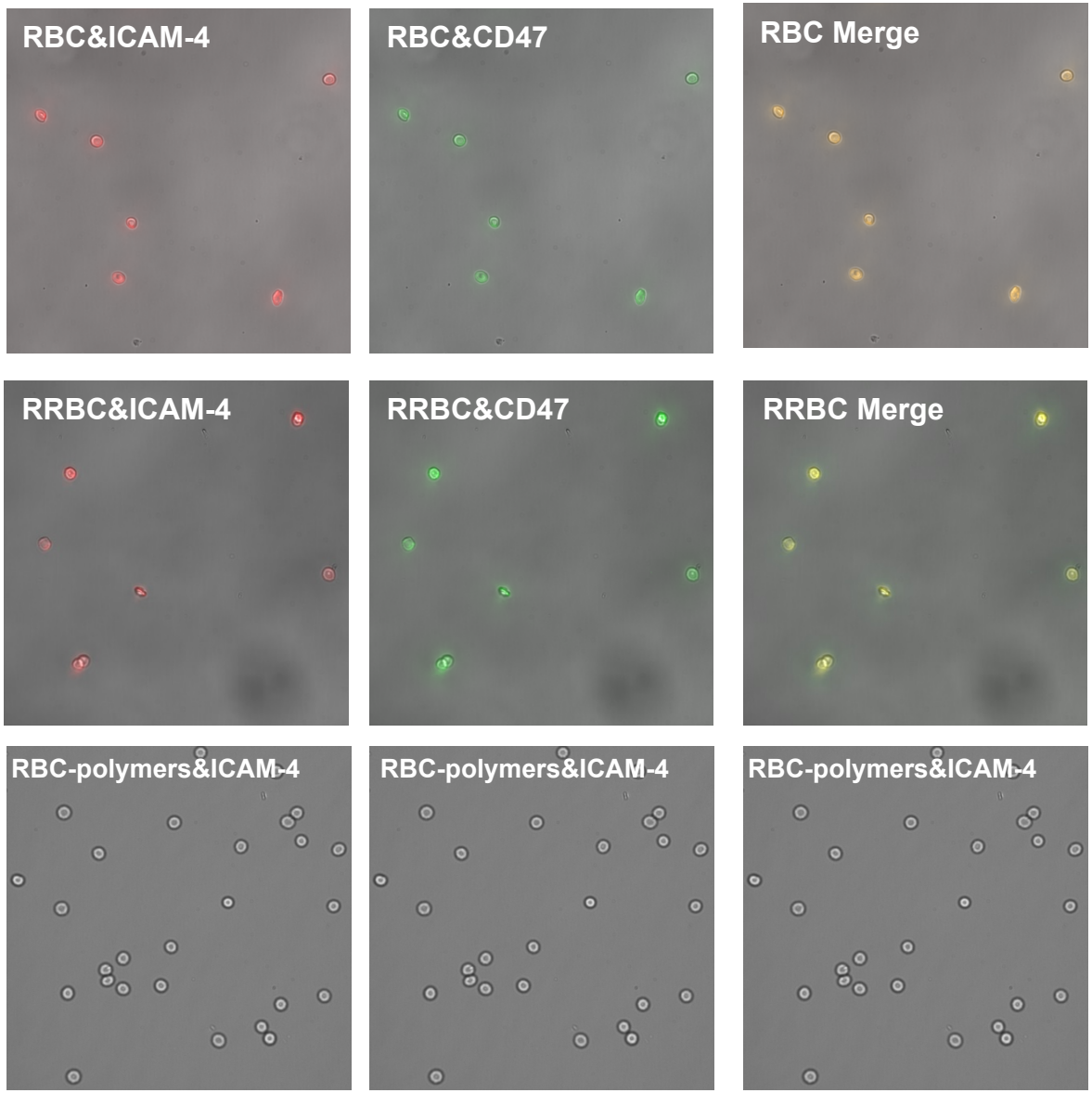
Right-side-out-membrane orientation of the rebuilt RBC is important in maintaining the same surface property as the native RBC.

CD47 and ICAM-4 'Self-Recognition' antigens preserved on RRBCs



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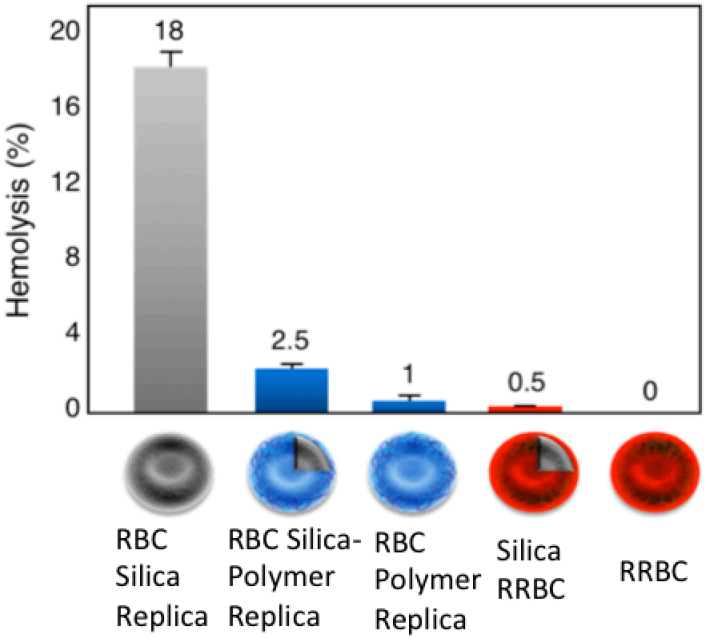
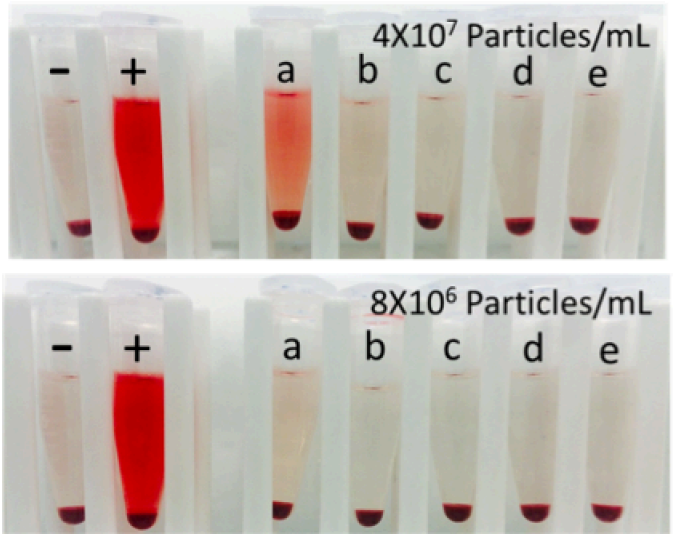
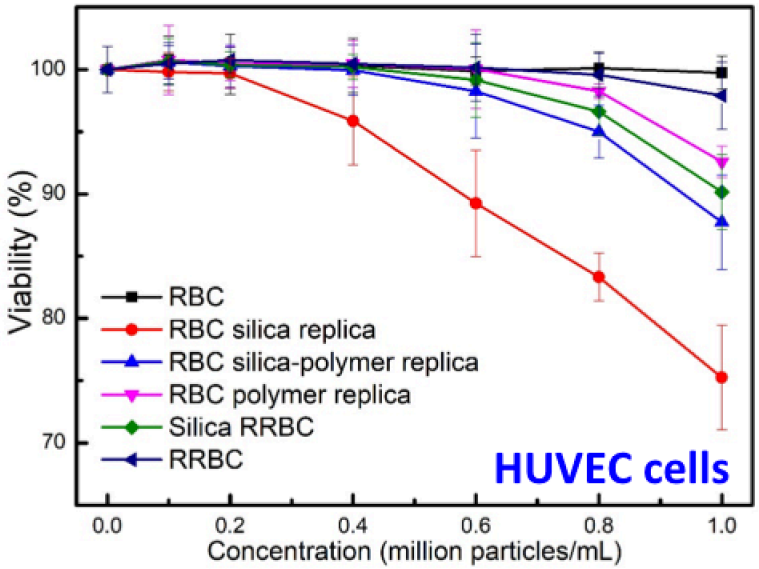
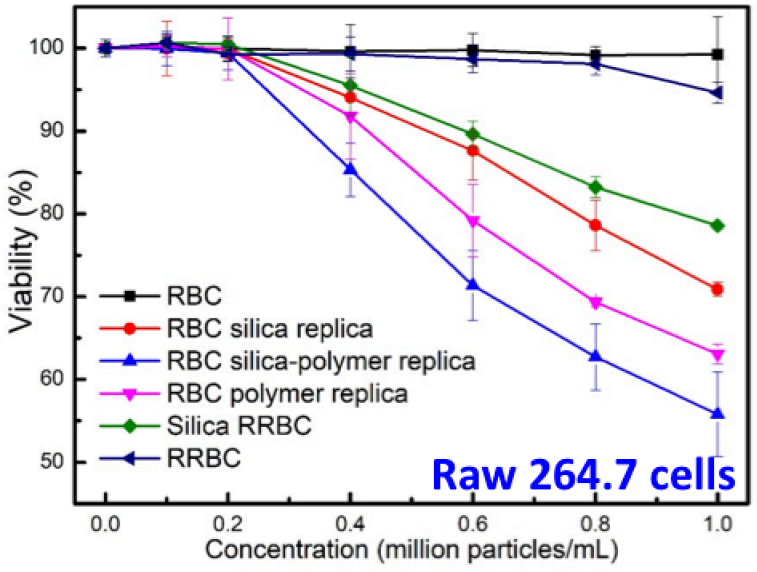
RRBC is biocompatible with native RBCs, endothelial cells, and macrophages







Human Umbilical Vein Endothelium (HUVEC) Cells



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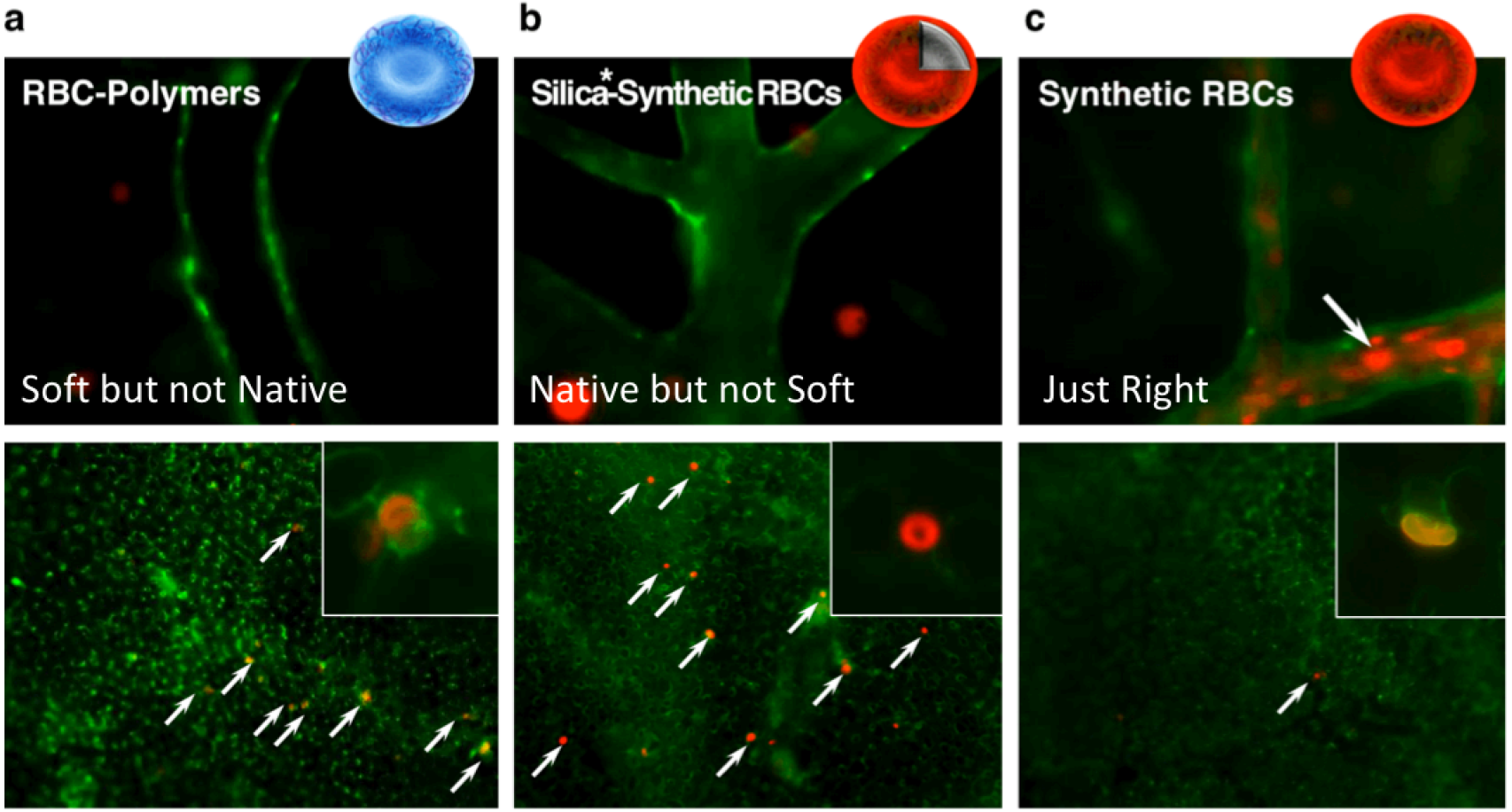
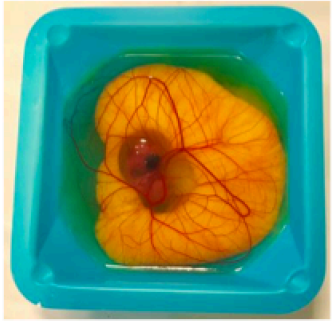
RRBCs readily circulate in the chicken embryo vasculature



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



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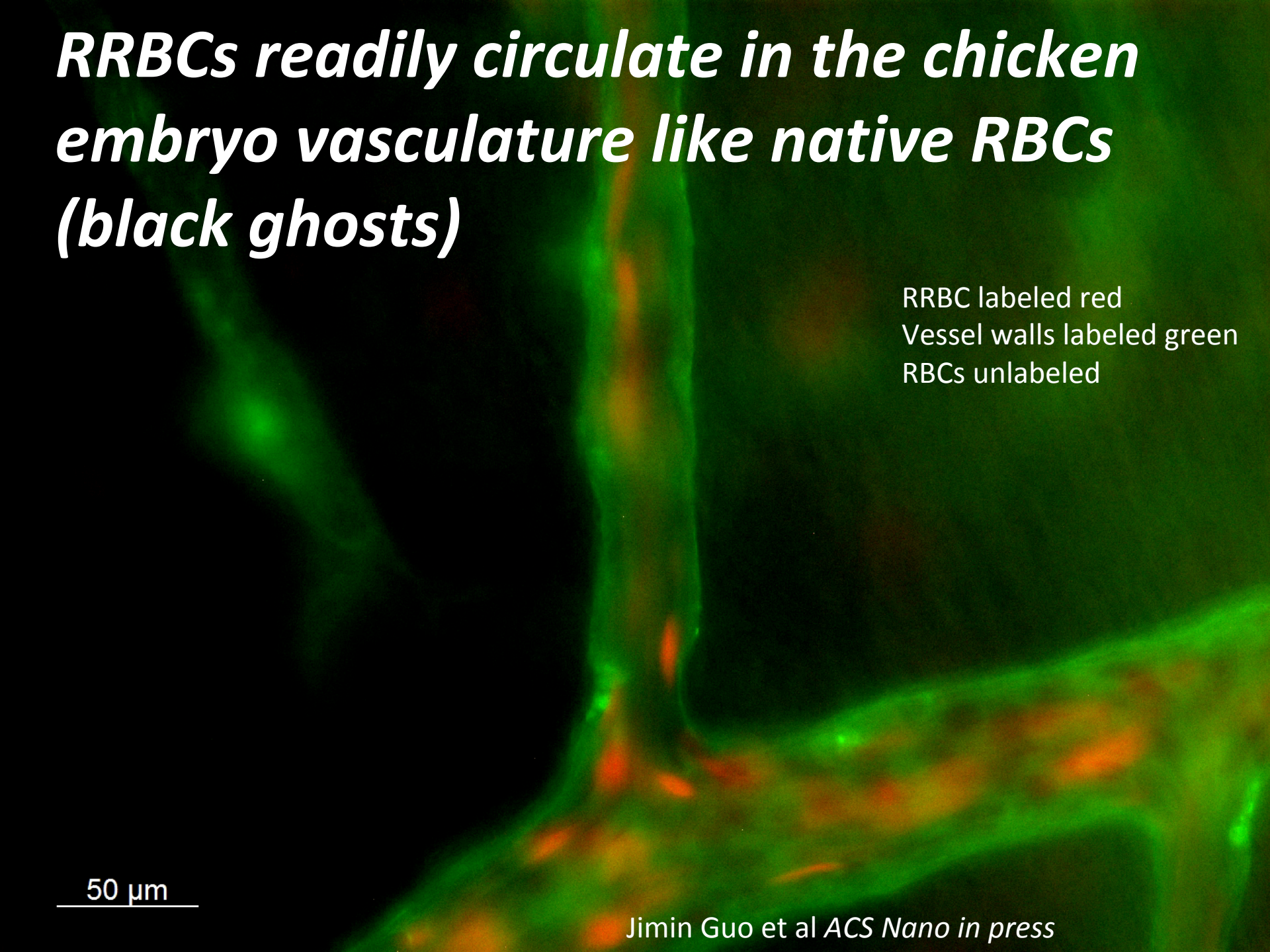
RRBCs readily circulate in the chicken embryo vasculature like native RBCs (black ghosts)



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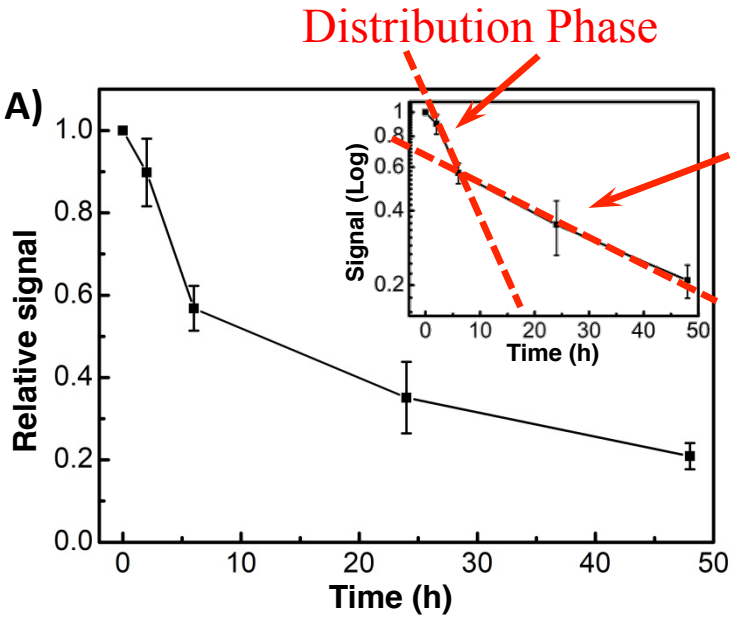
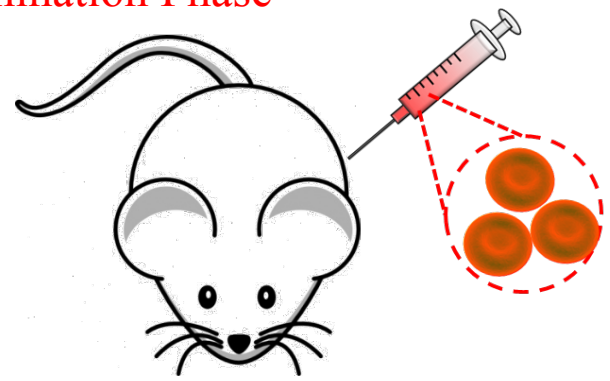
Elimination half-life of the RRBC was 41.8 h

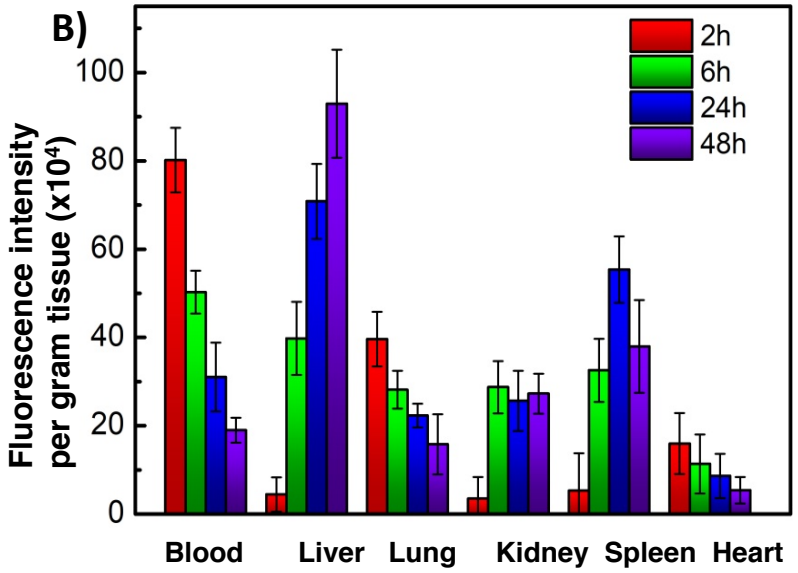


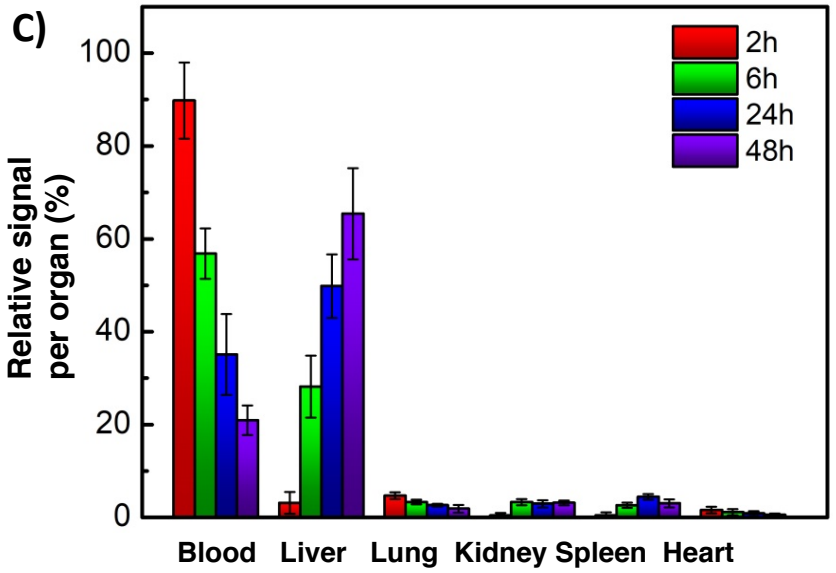
Elimination half-life was calculated as

$t_{1/2}=\ln(2)/\beta \quad C(t)=Ae^{-\alpha t}+Be^{-\beta t}$



In vivo circulation

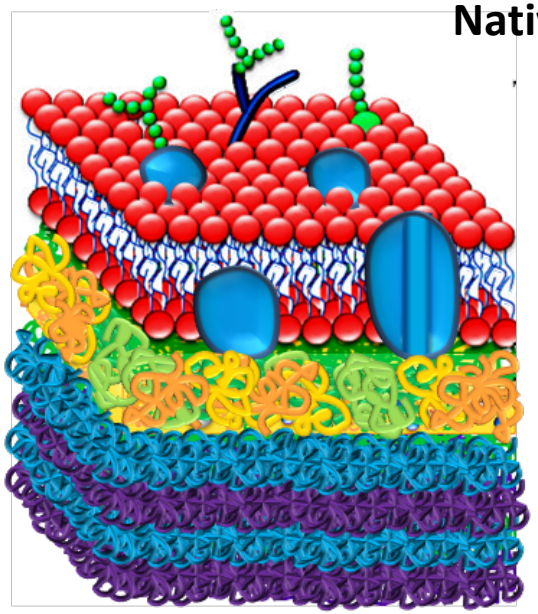


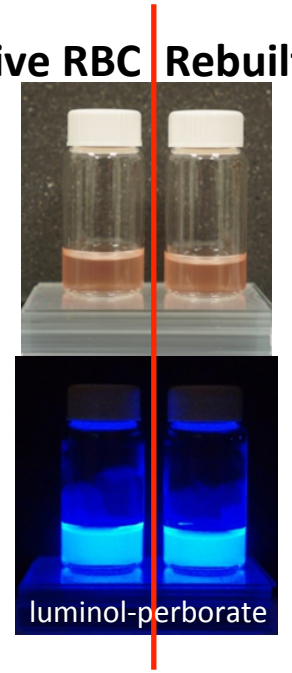


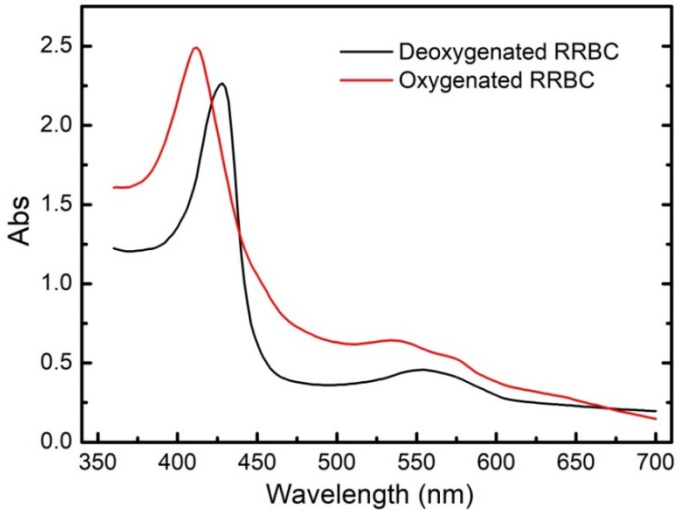
RBC ghosts derived from mouse model – Why 42h? How do we further increase circulation?

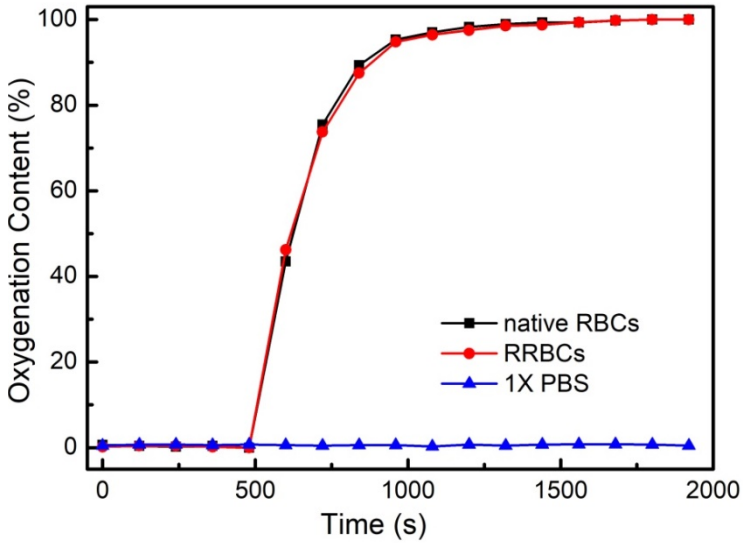
RRBCs can be loaded with Hemoglobin

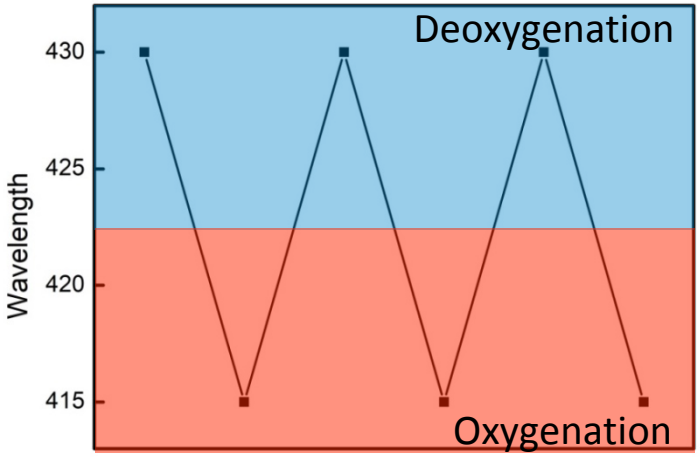
Chitosan layer incubated with hemoglobin prior to RBC ghost fusion











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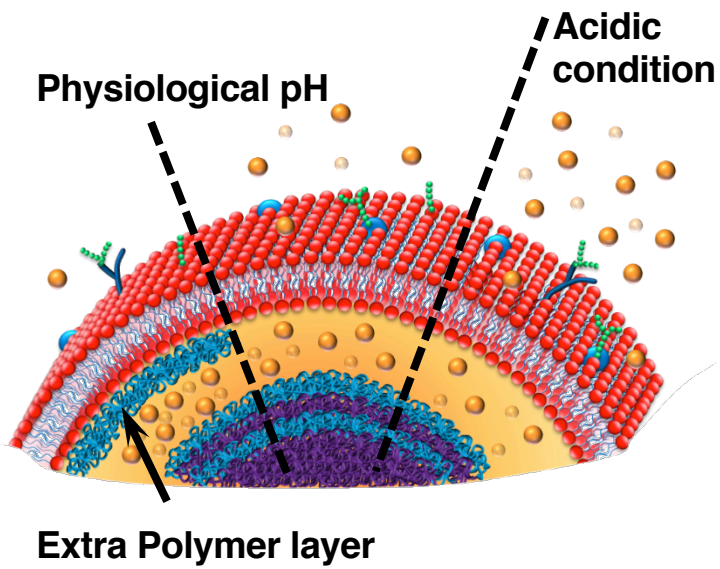
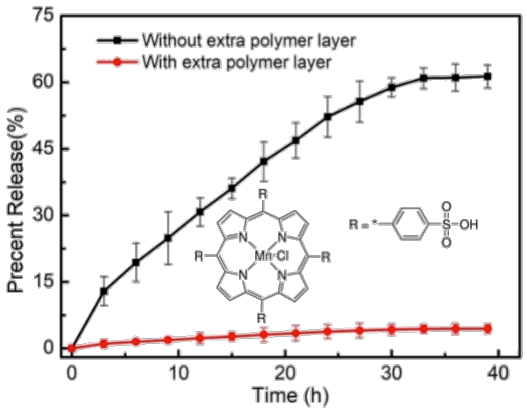
RRBC can be loaded with functional cargos and be magnetically sequestered from fluids

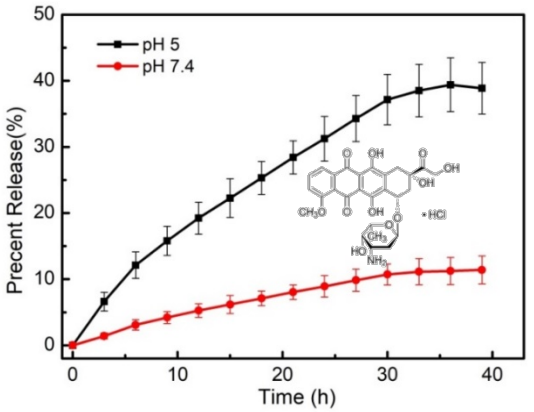


MRI contrast agent



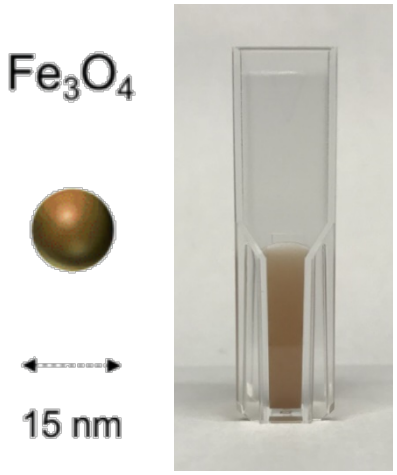
Porphine Manganese
3.5 µg/million particles

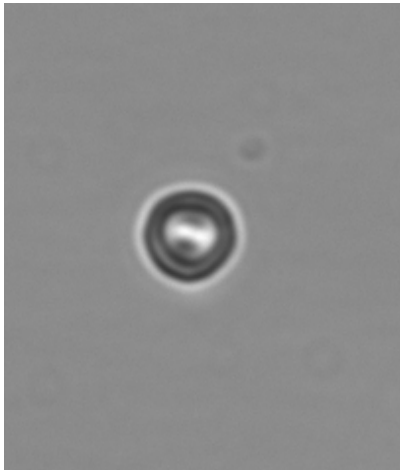
Anticancer drug

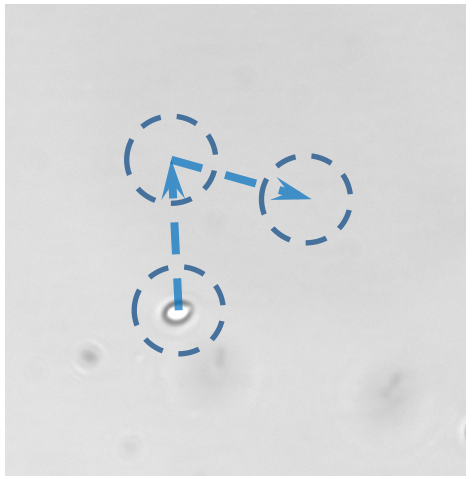


Doxorubicin
5.5 µg/million particles

Magnetic rebuilt RBCs







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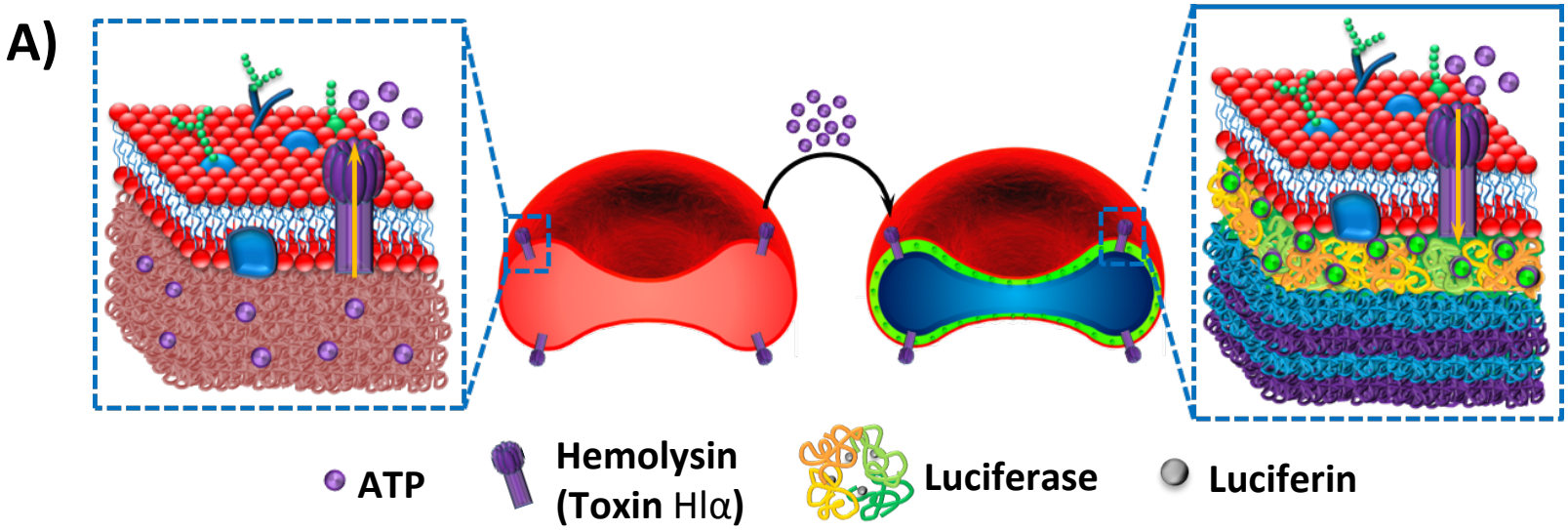
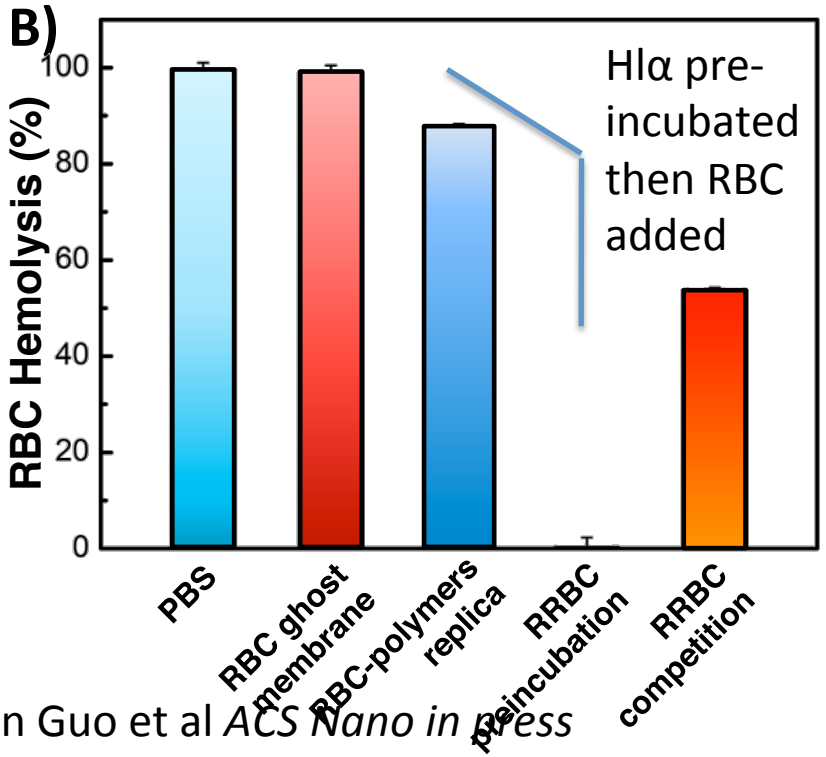
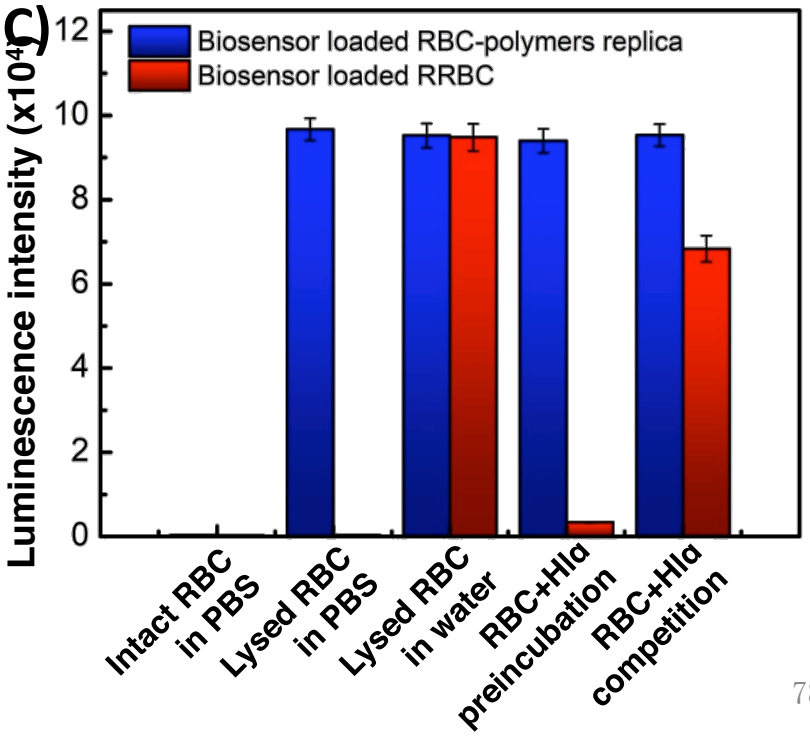
RRBCs serve as long circulating detox and sensing agents







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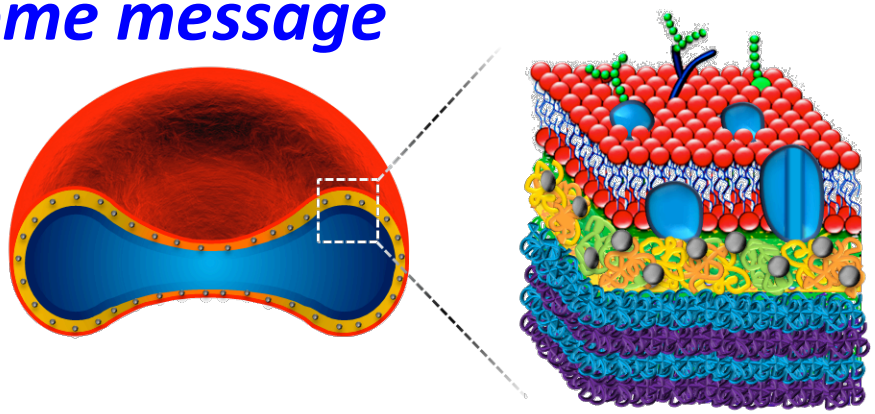
Take home message



MEMBRANE
Self-antigens & Immune-evasive

FUNCTIONAL CARGO
Cargo delivery & Bio-detection

POLYMER CORE
Biconcave shape & Deformability

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.***

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

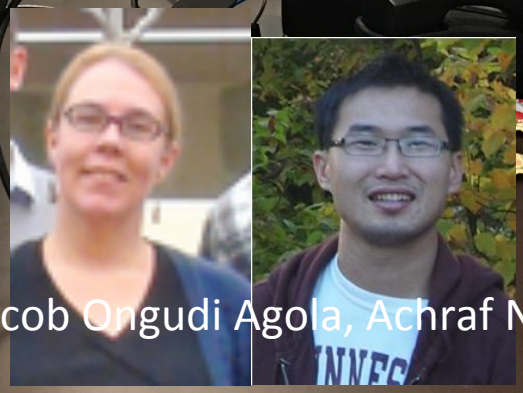
•Preserved biomolecular structure (FTIR) and functionality? (with caveat of fixation)

-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



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