CELLULAR MODIFICATION AND ARTIFICIAL CELL CONSTRUCTION

Jimin Guo
University of New Mexico

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Jimin Guo
Candidate

Chemical and Biological Engineering
Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

C. Jeffrey Brinker, Chairperson

Bryan Kaehr

Nick Carroll

Eva Chi

Atul Parikh
CELLULAR MODIFICATION AND ARTIFICIAL CELL CONSTRUCTION

by

JIMIN GUO

B.E. Bioengineering, Sichuan University, 2011
M.E. Biological and Chemical Engineering, Sichuan University, 2014

DISSERTATION
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Engineering

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DEDICATION

This dissertation is dedicated to my parents.

My mom and dad
who give me
the warmest love,
encouragement,
and endless support.
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C\textsc{ellular} M\textsc{odification} and A\textsc{rtificial} C\textsc{ell} C\textsc{onstruction}

by

J\textsc{imin} G\textsc{uo}

B.E. Bioengineering, Sichuan University, 2011

M.E. Biological and Chemical Engineering, Sichuan University, 2014

Ph.D. Engineering, University of New Mexico, 2019

A\textsc{bstract}

With all its complexities and different biofunctionalities, a cell is the basic structural and functional unit of all living organisms with the exception of viruses. In this dissertation, I demonstrated gain of function strategies of modifying mammalian cells using silicification and modular nanoparticle (NP) exoskeletons. In addition, I used a modular design concept to build a multifunctional artificial RBC system which can mimic the unique characteristics (e.g. shape, flexibility, the ability to carry oxygen, and long circulation times) of the native RBCs. Together, these cell modification or artificial cell construction strategies are expected to generate new mammalian cell-like structures with potential applications in biosensor, bioreactors and cell based therapy developments, as well as providing new platforms for carrying out cell biology studies.

Chapter One is a scientific introduction and discussion focusing on the development and challenges of whole cell bioactivities preservation, cell in shell modification and artificial cell construction.
Chapter Two describes silica cell replication method to preserve biofunctionalities of proteins in a silicified cell and maintains them in a “lifelike” state for a long period of time. In this chapter, we have shown that we can use antibody and nanoparticle probes to determine the accessibility and preservation of these biomarkers displayed on both the extracellular membrane and intracellular compartments throughout the course of silicification, dehydration, and desilicification. We have also shown that the activities of esterase, phosphatase (acid and alkali), and protease enzymes in hybrid silica biocomposites are preserved both in the short and long-term.

Chapter Three introduces the concept of ‘SupraCells’, which are living mammalian cells encapsulated and protected within functional modular nanoparticle-based exoskeletons. Exoskeletons are generated within seconds through immediate interparticle and cell/particle complexation that abolishes the macropinocytotic and endocytotic nanoparticle internalization pathways that occur without complexation. SupraCell formation was shown to be generalizable to wide classes of nanoparticles and various types of cells. It induces a spore-like state, wherein cells do not replicate or spread on surfaces but are endowed with extremophile properties, e.g., resistance to osmotic stress, reactive oxygen species, pH, and UV exposure, along with abiotic properties like magnetism, conductivity, and multi-fluorescence. Upon de-complexation cells return to their normal replicative states. SupraCells represent a new class of living hybrid materials with a broad range of functionalities.
Chapter Four further extend the functional modular nanoparticle-based exoskeletons encapsulation strategy to RBCs, so called ‘Armored Red Blood Cells’ (Armored RBCs). We found that Armored RBCs preserve the original properties of RBCs, inherit those of MOFs NP and show enhanced resistance against external stressors. By modifying the physicochemical properties of MOF NPs, Armored RBCs provide diagnostic properties like blood nitric oxide sensing or contrast for multimodal imaging. The synthesis of Armored RBCs is straightforward, reliable and reversible allowing for stepwise disassembly into distinct building blocks. Its general applicability allows its application to not only any kind of MOF NPs but potentially also to any cell type. We believe the presented concept enlarges the tool box of hybrid nanomedicines to unlock their potential for different fields ranging from biomedical imaging detection and therapy to targeted 3D micropatterning in cells and even personalized medicine.

Chapter Five introduces a modular design concept to build a multifunctional artificial RBC system. Here a micron-sized multifunctional RBC replica (rebuilt RBC) is designed and constructed using the successive steps of silica bio-replication, calcination, polyion polymer layer-by-layer deposition, desilicification, and fusion of RBC membrane ‘ghosts’. The rebuilt RBC displays biconcave shape, deformability, zero hemolytic activity, and vascular flow in ex ovo chick embryo and in vivo mouse models. The rebuilt RBC is also loadable with various cargos including hemoglobin for oxygen transport, contrast agents for magnetic target localization or magnetic resonance imaging applications, therapeutic small molecules, and a biosensor for pore-forming toxin detection. Overall, rebuilt RBCs
represent a new, robust, long circulating synthetic platform for use in therapy bio-
detection, and imaging as well as a unique tool to advance our understanding of complex life processes.

To conclude, Chapter Six reviews my thesis work and highlights the advances I have made in the fields of cellular modification and artificial cell construction. In addition, possible future directions are also described.
CHAPTER 1

INTRODUCTION

1.1 Overview

1.2 Challenges in cell modification for whole cell bioactivities preservation

1.3 Challenges in cell-in-shell modification

1.4 Challenges in artificial cell construction

1.5 References

CHAPTER 2

Whole cell biofunctional preservation using silica replication process

2.1 Introduction

2.2 Results and Discussion

2.2.1 Silicification

2.2.2 Antigen preservation

2.2.3 Enzyme activity preservation

2.3 Conclusion

2.4 Materials and Methods

2.4.1 Materials

2.4.2 Cell culture and chemical fixation

2.4.3 Silicification and desilicification of mammalian cells

2.4.4 Immunofluorescence staining

2.4.5 Enzymatic activity study

2.5 References

CHAPTER 3
SupraCells: Living Mammalian Cells Protected within Functional Modular Nanoparticle-Based Exoskeletons

3.1 Introduction

3.2 Results and Discussion

3.2.1 Formation and characterization of SupraCells

3.2.2 Enhanced resistance of SupraCells against endo- and exogenous stimuli

3.2.3 Enhanced mechanical resistance of SupraCells

3.2.3 SupraCell-properties based on modular functional NP-based exoskeletons

3.3 Conclusion

3.4 Materials and Methods

3.4.1 General information

3.4.2 Nanoparticles synthesis

3.4.3 Cell culture

3.4.4 SupraCell construction

3.4.5 Cell viability test

3.4.6 SupraCell shell controlled disassembly or dissociation

3.4.7 Cell culture and proliferation test

3.4.8 SupraCell mechanical characterization

3.4.9 SupraCell Permeability test

3.4.10 SupraCell Cytoprotection test

3.4.11 Phagocytosis assay

3.4.12 SupraCell tolerance at harsh conditions

3.4.13 SupraCell-Modular nanoparticle superassembly

3.4.14 SupraCell-Magnetic manipulation

3.4.15 SupraCell-In situ NO sensing

3.4.16 SupraCell Conductivity measurement

3.5 References

CHAPTER 4

Armored red blood cells: multifunction integration through modular superassembly of hybrid nanoparticle-based building blocks
4.1 Introduction ................................................................................................................. 63
4.2 Results and Discussion ............................................................................................... 67
  4.2.1 Modular super-assembly of Armored RBCs ............................................................ 67
  4.2.2 Armored RBCs show enhanced resistance against external stressors 71
  4.2.3 Assessment of Armored RBCs with respect to their oxygen carrier capability, ex ovo and in vivo circulation and biodistribution .............................. 75
  4.2.4 Multifunctional Armored RBCs construction ......................................................... 81
4.3 Conclusion .................................................................................................................. 85
4.4 Materials and Methods .............................................................................................. 87
  4.4.1 General information .............................................................................................. 87
  4.4.2 Nanoparticles synthesis ......................................................................................... 88
  4.4.3 RBC Purification ..................................................................................................... 91
  4.4.4 Armored RBC construction .................................................................................... 91
  4.4.5 Hemolysis assay .................................................................................................... 92
  4.4.6 Antibody-mediated agglutination .......................................................................... 93
  4.4.7 Tolerance against ion strength .............................................................................. 93
  4.4.8 Tolerance against detergent .................................................................................. 93
  4.4.9 Tolerance against toxic nanoparticles ................................................................... 94
  4.4.10 Cryopreservation and cell recovery ..................................................................... 94
  4.4.11 Chemiluminescence ............................................................................................ 95
  4.4.12 Capability of reversibly binding oxygen .............................................................. 95
  4.4.13 Test of vascular flow in Ex ovo chick embryos ...................................................... 96
  4.4.14 In vivo studies on pharmacokinetics and biodistribution ...................................... 97
  4.4.15 Armored RBC shell controlled destruction .......................................................... 98
  4.4.16 NO sensing .......................................................................................................... 98
  4.4.17 Armored RBC magnetic manipulation ................................................................. 99
  4.4.18 Armored RBC modular nanoparticles super-assembly .......................................... 99
4.5 References .................................................................................................................. 100
CHAPTER 5 ......................................................................................................................... 104
Biomimetic Rebuilding of Multifunctional Red Blood Cell: Design Using Functional Modules .............................................................................................................. 104
  5.1 Introduction .............................................................................................................. 105
### 5.2 Results and Discussion

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.1 Construction of Rebuilt RBCs</td>
<td>107</td>
</tr>
<tr>
<td>5.2.2 Deformability of Rebuilt RBCs</td>
<td>110</td>
</tr>
<tr>
<td>5.2.3 Surface properties of Rebuilt RBCs</td>
<td>114</td>
</tr>
<tr>
<td>5.2.4 Biocompatibility and circulation properties of Rebuilt RBCs</td>
<td>115</td>
</tr>
<tr>
<td>5.2.5 Oxygen carrying capability of Rebuilt RBCs</td>
<td>120</td>
</tr>
<tr>
<td>5.2.6 Multifunctional Rebuilt RBCs construction</td>
<td>122</td>
</tr>
</tbody>
</table>

### 5.3 Conclusion

<table>
<thead>
<tr>
<th>Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
</tr>
</tbody>
</table>

### 5.4 Materials and Methods

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4.1 Materials</td>
<td>129</td>
</tr>
<tr>
<td>5.4.2 Purification of RBCs</td>
<td>130</td>
</tr>
<tr>
<td>5.4.3 Preparation of silica RBC replicas</td>
<td>130</td>
</tr>
<tr>
<td>5.4.4 Preparation of RBC-polymer replica</td>
<td>131</td>
</tr>
<tr>
<td>5.4.5 Preparation of RBC-membrane-derived ghosts</td>
<td>131</td>
</tr>
<tr>
<td>5.4.6 Preparation of rebuilt RBC (RRBC) particles</td>
<td>132</td>
</tr>
<tr>
<td>5.4.7 Zeta potential measurements</td>
<td>132</td>
</tr>
<tr>
<td>5.4.8 Scanning electron microscopy (SEM) imaging</td>
<td>132</td>
</tr>
<tr>
<td>5.4.9 Confocal microscopy imaging</td>
<td>133</td>
</tr>
<tr>
<td>5.4.10 Microfluidic blood capillary model and experiments</td>
<td>133</td>
</tr>
<tr>
<td>5.4.11 Antibody-mediated agglutination assay</td>
<td>134</td>
</tr>
<tr>
<td>5.4.12 Immunofluorescence staining</td>
<td>134</td>
</tr>
<tr>
<td>5.4.13 Hemolysis assay</td>
<td>135</td>
</tr>
<tr>
<td>5.4.14 Cell viability assay</td>
<td>135</td>
</tr>
<tr>
<td>5.4.15 Test of vascular flow in ex ovo chick embryos</td>
<td>136</td>
</tr>
<tr>
<td>5.4.16 Pharmacokinetics and biodistribution studies</td>
<td>136</td>
</tr>
<tr>
<td>5.4.17 Hemoglobin loading</td>
<td>137</td>
</tr>
<tr>
<td>5.4.18 Chemiluminescence assays</td>
<td>138</td>
</tr>
<tr>
<td>5.4.19 Assay of the reversible binding of oxygen</td>
<td>138</td>
</tr>
<tr>
<td>5.4.20 Loading and release kinetics of small molecules</td>
<td>139</td>
</tr>
<tr>
<td>5.4.21 Magnetic iron oxide nanoparticles synthesis</td>
<td>140</td>
</tr>
<tr>
<td>5.4.22 Loading of magnetic nanoparticles</td>
<td>141</td>
</tr>
<tr>
<td>5.4.23 Confirmation of the Fe3O4 nanoparticle loading</td>
<td>141</td>
</tr>
</tbody>
</table>
5.4.24 Quantification of toxin hemolytic activity ........................................ 141
5.4.25 Toxin absorption study in vitro ..................................................... 142
5.4.26 Toxin neutralization in vitro ......................................................... 142
5.4.27 Luciferase-luciferin biosensor loading ............................................ 143
5.4.28 Luciferase activity assay ............................................................... 143
5.4.29 Evaluation of Pore-forming toxin sensor ....................................... 144

5.5 References .................................................................................. 145

CONCLUSIONS AND FUTURE DIRECTIONS ........................................... 149

6.1 Conclusions .............................................................................. 150

6.3 Future Directions ....................................................................... 153

SUMMARY OF CONTRIBUTIONS ......................................................... 15555

APPENDIX 1 Supporting Information for Chapter 2 ......................... 158

APPENDIX 2 Supporting Information for Chapter 3 ....................... 161

APPENDIX 3 Supporting Information for Chapter 4 ....................... 192

APPENDIX 4 Supporting Information for Chapter 5 ....................... 199
# LIST OF FIGURES

| Figure 2.1 | Silica diffusion in the silicified cells | 12 |
| Figure 2.2 | Schematic of silification process | 13 |
| Figure 2.3 | FTIR and ICP-OES of silicified cells | 14 |
| Figure 2.4 | Schematic of cell and biomarker binding position | 16 |
| Figure 2.5 | Immunostaining of silicified cells | 17 |
| Figure 2.6 | Enzyme activity of silicified cells | 19 |
| Figure 2.7 | Enzyme activity preservation of silicified cells | 22 |
| Figure 3.1 | Formation and characterization of SupraCells | 32 |
| Figure 3.2 | Resistance of SupraCells | 36 |
| Figure 3.3 | Mechanical resistance of SupraCells | 40 |
| Figure 3.4 | Modular functional NP-based exoskeletons | 43 |
| Figure 4.1 | Modular functionalization of RBCs with MOF nanobuilding blocks | 65 |
| Figure 4.2 | Armored RBC structure characterization | 70 |
| Figure 4.3 | Resistance of Armored RBC | 73 |
| Figure 4.4 | Armored RBC oxygen carrier capability and ex ovo circulation | 76 |
| Figure 4.5 | Armored RBCs circulation and biodistribution | 80 |
| Figure 4.6 | Multifunctional Armored RBCs | 82 |
| Scheme 5.1 | Schematic of rebuilt RBC | 106 |
| Figure 5.1 | Formation and characterization of rebuilt RBC | 108 |
| Figure 5.2 | Deformability of rebuilt RBCs | 112 |
| Figure 5.3 | Rebuilt RBCs in CAM | 117 |
| Figure 5.4 | Oxygen carrier capability and circulation property of rebuilt RBCs | 120 |
| Figure 5.5 | Multifunctional rebuilt RBCs | 122 |
LIST OF ABBREVIATIONS

\( \zeta \):  Zeta Potential
\( \equiv \text{Si-O}^- \):  Deprotonated Silanol Group
\( \equiv \text{Si-OH} \):  Silanol Group
APTES:  3-aminopropyltriethoxysilane
BET:  Brunauer-Emmet-Teller
CAM:  Chorioallantoic Membrane
COX IV:  Mitochondrial Cytochrome C Oxidase Subunit IV
CM-H2DCFDA:  Chloromethyl Derivative 2',7'-Dichlorodihydrofluorescein Diacetate
DAPI:  4',6-Diamidino-2-Phenylindole, Dihydrochloride (Blue Nuclear Stain)
DLS:  Dynamic Light Scattering
DMEM:  Dulbecco's Modified Eagle Medium
DMF:  N,N-Dimethyl Formamide
DMSO:  Dimethyl Sulfoxide
DOX:  Doxorubicin
EGFR:  Epidermal Growth Factor Receptor
FBS:  Fetal Bovine Serum
FTIR:  Fourier Transform Infrared Spectroscopy
FTIR:  Fourier Transform Infrared Spectroscopy
Lamp-1:  Lysosomal-associated Membrane Protein 1
MRI:  Magnetic Resonance Imaging
MSNP:  Mesoporous Silica Nanoparticle
MUP:  4-Methylumbelliferyl Phosphate
Pdi:  Polydispersity Index
PEG:  Polyethylene glycol
PNPP:  p-Nitrophenyl Phosphate
RBC:  Red blood cell
RITC:  Rhodamine B Isothiocyanate
ROS:  Reactive Oxygen Species
SD:  Standard Deviation
TEOS:  Tetraethyl Orthosilicate
TEM:  Transmission Electron Microscopy
CHAPTER 1

INTRODUCTION
1.1 Overview

Cells have been studied for several centuries as the basic structural and functional units of all known living organisms, consisting of a complex hierarchical architecture (DNA, protein, organelle, and whole cell).[1] Inside every cell, deoxyribonucleic acid (DNA) encodes the information needed to specify the cell, stores hereditary information and regulates the expression of this information,[2] while proteins, transcribed and translated from the DNA, carry out the majority of basic cellular activities that ensure the overall survival of the living organism.[1]

When proteins accumulate in specific regions of the cell and interact with other proteins or biomolecules, they can form large assemblies called organelles that carry out particular and specialized physiological functions. With the construction of various organelles, a cell can survive and perform its particular specialized tasks.

1.2 Challenges in cell modification for whole cell bioactivities preservation

Since DNA and proteins are the cornerstones of cellular biofunctions in all living creatures, they have received significant attention in both research and industrial applications. For example, DNA–based bioinformation storage medium is being used to identify the biological origin in genetics, criminology, and food industry.[2] DNA is also being used as a long-term data-storage medium replacing traditional optical or magnetic storage technologies.[2,3] Proteins as molecular devices where biological function is exerted, are being used in biosensors and bioreactors.[4] Some immunogenic proteins are also widely used as antigens for vaccine research applications.[5] In spite of these important and diverse applications, DNA and
proteins tend to be delicate and their overall stability still remains a matter of concern to many users. While it might be easy to preserve the integrity of purified biomolecules using common methods such as low temperature freezing, long-term preservation of the functions of DNA and proteins in a cell is still problematic due to time dependent degradation. Even the often-used low temperature freezing presents its own sets of challenges such as the need for continuous cold storage which is not cost effective in the long-term.\(^6\) Moreover, thawing or equilibrating frozen biological samples to room temperature can lead to unfavorable ice crystal-biomolecule interactions which often reduces the degree of biomolecular functions.\(^6\) Chemical fixation is another approach commonly used to preserve biological materials as close to their natural states as possible by preventing autolysis or putrefaction at room temperature. In particular, formaldehyde, which is one of the preferred fixatives, tends to preserve the secondary structure of proteins and protects significant amounts of the tertiary structure as well stabilizing proteins and most of their activities.\(^7\) However, when stored for a long time, formaldehyde fixative is susceptible to oxidation to formic acid which technically renders fixation ineffective.\(^7\) Moreover, increasing fixation time or storage time after fixation increases the risk of denaturation of biomolecules and easy loss of bioactivities. Thus, inspired by siliceous exoskeletons of unicellular diatoms, that tolerate harsh environmental conditions by using their silicified molecular framework, the biomimetic silicification process becomes an attractive strategy for preserving the integrity of biomolecules.\(^8\) Traditionally, biomimetic cell silicification has been mainly used to encapsulate cells in siliceous exoskeletons.
without any protection of the intracellular proteins from autolysis. [9] This calls for the development of better biomimetic cell silicification strategies that can preserve the functions of whole cell biomolecules.

1.3 Challenges in cell-in-shell modification

In the search for better methods of preserving the functional integrity of biomolecules in the cell, scientists have been interested in both the viability of whole cells and identifying means of adding new abiotic functions to the cells different from the original specializations. Inspired by spores, cell encapsulation or generating cell-in-shell structures or “artificial spores”, can provide a versatile chemical toolbox for enhancing cell tolerance against various harmful stressors, controlling cellular behaviors, and introducing abiotic photochemical, electrochemical, magnetic, and thermal properties for the utilization and manipulation of the cells.[4,10] Specifically, by mimicking the natural ‘sporulation’ process through the encapsulation of the cell within a durable artificial shell such as silica, titanium, gold, iron oxide, polymer, and metal–organic frameworks, cellular tolerance against external stressors (e.g., UV irradiation, heat, freezing, and enzymatic attack) can be greatly enhanced as well as the alteration of metabolic activities, as exemplified by the suppressed or retarded cell cycle progression of the encapsulated cells.[4] Through a biomimicking process termed as “germination”, the development of a cytoprotective shell with on-demand degradation such as a metal-phenolic network, can further promote well-controlled cell adhesion and proliferation through programmed shell formation and degradation.[10,11] In addition, the physicochemical properties of these artificial shell
materials can contribute to the endowed abiotic functions of nanocoated single-cells that living cells do not possess originally including: electrical conductivity, magnetism, photochemical, and stimulus-response functions. Although these cell-in-shell hybrids provide a fundamental research platform for single cell-based analysis and have potential applications in whole-cell based biocatalysts, previous studies in this field primarily focused on the feasibility demonstrations of the encapsulation process for sustaining cell viability, and the introduced functionalities were singularly functional but not multiplexed. Moreover, most of the studies were focused on living microbial systems, with few of them involving the more sensitive and delicate mammalian cells. For a mammalian cell whose membrane consists of labile lipid bilayers that are fluidic and highly susceptible to environmental changes, design of multi-functional degradable cytoprotective shell around the cell surface is still in its infancy and remains a big challenge.

1.4 Challenges in artificial cell construction

In their current state, cell-in-shell hybrids are already half-artificial/half-natural systems. This has inspired design and building of a fully artificial cell which can mimic biological cell functions. This kind of design is best illustrated by the red blood cell (RBC) of higher organisms, which even though is simple in structure (e.g. lacking a cell nucleus and most intracellular organelles) is proving to be a suitable target for biomimicking and rebuilding of functional artificial cells. RBC possesses three main unique characteristics; special shape, flexibility/deformability, ability to carry oxygen, and long circulation times, that are the main biomimetic focal points. The biconcave discoidal shape of RBCs
provides a favorable surface area-to-volume ratio and allows RBCs to undergo remarkable deformations while mechanical flexibility allows them to pass through restricted capillaries smaller than their own diameter (7 μm), a feature unmatched by typically stiff and spherical synthetic particles.\cite{12-14} The hemoglobin contained in RBCs facilitates oxygen transport from the lungs to the cells of the body through the formation of an oxyhemoglobin complex.\cite{14} This function is aided by different molecular biomarkers on the RBC membrane that ensure self-recognition and evasion of the macrophages of the immune system, leading to long circulation times.\cite{14-16} There have been a few precedential attempts to mimic the key structural and functional features of RBCs to generate artificial replica materials. For example, Mitragotria, et al.\cite{14} developed synthetic biomaterial particles that closely mimic the shape, flexibility, and the ability to carry oxygen as natural red blood cells. Similarly, DeSimonea, et al.\cite{16} explored the effect of mimicking RBC’s shape and flexibility on the circulation time and biodistribution characteristics of an RBC-like material. While these investigations presented encouraging findings, their main shortcoming is the predominant focus on one or two aspects of the RBC’s unique characteristics, rather than mimicking a broad spectrum of RBC’s properties to generate a potentially multifunctional RBC mimic system. In addition, none of these previous studies ever considered the unique properties of the RBC membrane and their potential impacts on the long-term circulation times. Thus, in this study, I seek to rebuild an artificial RBC or RBC mimic particle which can fully mimic broad properties of the native RBC as well as perform additional abiotic functions not inherent to the native RBCs.
1.5 References


CHAPTER 2

Whole cell biofunctional preservation using silica replication process
2.1 Introduction

Cells have been studied for several centuries as the basic structural and functional units of all known living organisms, consisting of a complex layered architecture (DNA, protein, organelle, and whole cell).[1] Inside every cell, proteins carry out the majority of basic cellular activities and perform particular and specialized physiological functions, that ensure the overall survival of the living organism.[1]

Since proteins are the cornerstones of cellular biofunctions in all living creatures, they have received significant attention in both research and industrial applications. For example, proteins as molecular devices where biological function is exerted, are being used in biosensors and bioreactors.[2] Some immunogenic proteins are also widely used as antigens for vaccine research applications.[3] In spite of these important and diverse applications, proteins tend to be delicate and their overall stability still remains a matter of concern to many users. While it might be easy to preserve the integrity of purified biomolecules using common methods such as low temperature freezing, long-term preservation of the functions of proteins in a cell is still problematic due to time dependent degradation. Even the often-used low temperature freezing presents its own sets of challenges such as the need for continuous cold storage which is not cost effective in the long-term.[4] Moreover, thawing or equilibrating frozen biological samples to room temperature can lead to unfavorable ice crystal-biomolecule interactions which often reduces the degree of biomolecular functions.[4] Chemical fixation is another approach commonly used to preserve biological materials as close to their natural states as possible by preventing autolysis or putrefaction at room temperature. In particular,
formaldehyde, which is one of the preferred fixatives, tends to preserve the secondary structure of proteins and protects significant amounts of the tertiary structure as well stabilizing proteins and most of their activities.\[^{[5]}\] However, when stored for a long time, formaldehyde fixative is susceptible to oxidation to formic acid which technically renders fixation ineffective.\[^{[5]}\] Moreover, increasing fixation time or storage time after fixation increases the risk of denaturation of biomolecules and easy loss of bioactivities. Thus, inspired by siliceous exoskeletons of unicellular diatoms, that tolerate harsh environmental conditions by using their silicified molecular framework, the biomimetic silification process becomes an attractive strategy for preserving the integrity of biomolecules.\[^{[6]}\] Traditionally, biomimetic cell silification has been mainly used to encapsulate cells in siliceous exoskeletons without any protection of the intracellular proteins from autolysis.\[^{[7]}\] This calls for the development of better biomimetic cell silification strategies that can preserve the functions of whole cell biomolecules.

Unlike traditional biomimetic cell silification approach, silica bio-replication or freezing cells in silica, forms not only siliceous exoskeletons but also a siliceous cytoskeleton to preserve inter- and intracellular heterogeneity from the nano- to macroscale even after drying.\[^{[7]}\] This new self-limiting biomolecular surface-directed silica assembly process can address some of the weaknesses of the traditional biomimetic cell silification which has been mainly used to encapsulate cells in siliceous exoskeletons,\[^{[6]}\] and cannot prevent intracellular proteins from autolysis. Based on the salient features of silica cell replication such as tolerance of harsh environmental conditions, I hope to demonstrate the versatility of silica
cell replication as a novel approach that can preserve biofunctionalities of whole cellular proteins in the silicified cell and keep them as “lifelike” as possible for a long period of time.

2.2 Results and Discussion

2.2.1 Silicification

Preparation of the hybrid silica biocomposite material involves deposition and condensation of silicic acid (Si(OH)$_4$) onto fixed biological interfaces under mild acidic conditions (pH 3.0).

![Confocal microscopy images of 30 mins silicification with rhodamine-silane dye, indicating the silica formed not only on the cell membrane but also inside cells.](image)

**Figure 2.1.** Confocal microscopy images of 30 mins silicification with rhodamine-silane dye, indicating the silica formed not only on the cell membrane but also inside cells.
Specifically, silicic acid precursor tetramethyl orthosilicate (TMOS) could quickly hydrolysis and form silicic acid in mild acidic water solution. This acidic condition could suppress silica self-condensation and maintain the silicic acid as monomer or oligomer states (Figure S2.1). In addition, at pH 3, where silicic acid monomers and oligomers are uncharged, silicic acid incorporates within the continuous hydrogen bonded water network encompassing extra- and intercellular surfaces (Figure 2.1) where it becomes locally concentrated and subsequently condensed via amino groups of the cellular biomolecules (Figure 2.2).

**Figure 2.2.** A) Schematic of silicification. B) Schematic of self-assembled monolayers (SAM) structure. C) Atomic concentration of Si tested by X-ray photoelectron spectroscopy.

The FTIR and ICP-OES data also demonstrated silica content of silicified cells increased with the silicification time. (Figure 2.3A and 2.3B) In the first 60 min silicification, there was quick silica signal accumulation shown in *in situ* FTIR
test, but slow silica content shown. (Figure 2.3C and 2.3D) This result indicated that at pH 3, silicic acid quickly absorbed extra- and intercellular surfaces and then slowly condensed to form silica network. From $^{29}$Si CP/MAS NMR, the significant increase of single silanol (Q3)/siloxane bridges (Q4) signal showed after 6h silicification, further confirming the slow silica condensation during silicification process. (Figure S2.2)

Figure 2.3. A) In situ fourier-transform infrared (FTIR) spectroscopy and B) Si content test by ICP-OES of silicified cells during silicification process. C) Si-OH absorbance and D) Si content of silicified cells during silicification process.
Although the dimensional features of the macro- to nano-structure of cells, tissues, and organisms were preserved \cite{7,8}, the shape and feature of these silica biocomposites are completely preserved during the silicification process, but whether the biofunctionality of the biocomposites is also preserved remains to be explored.

\textbf{2.2.2 Antigen preservation}

Interestingly, although silicification occurred by protein catalyzed condensation at all biomolecular interfaces, it occurred without any perturbation of the protein assemblies as elucidated by FTIR. (\textbf{Figure 2.3A}) In this case, we assume that silicification could preservation of DNA and proteins in whole cells. To demonstrate this, molecular and antibody probes were used to investigate the accessibility and preserved biomolecular structures of cellular membrane and intracellular organelles in silicified A549 cells. Preliminary evidence showed that, even after cell silicification, recognition of surface and intracellular molecular biomarkers whose sizes range from 0.5-3.5nm \cite{9} (\textbf{Figure 2.4}) could still be achieved using antibodies and other molecular probes (\textbf{Figure 2.5}). However, it was noticed that for dehydrated or fully dried silicified cells, the antibody probes could not recognize the molecular biomarkers (\textbf{Figure 2.5 panel 3}), presumably due to further condensation/crosslinking of the silica layers promoted by the dehydration step that blocks diffusion of the larger antibody probes to intra and extracellular antigens. This was confirmed by the ability of the antibody to label the so-called ‘desilicified cells’ where the silica layer had been removed via etching with a buffered hydrofluoric acid solution that removes approximately 1nm.
silica/second (Figure 2.5 panel 4). Similar characteristics were observed with EGFR targeted nanoparticles (~50-nm in diameter, Figure S2.3), whereby the silica layer blocked the interactions of EGFR targeted nanoparticles with the surface-expressed epidermal growth factor receptor (EGFR), but antigen binding was restored after etching approximately 12-nm of silica.

![Figure 2.4. A) Schematic of cell and biomarker binding position. B) The size and staining position of biomarker.](image)

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Location of biomarker target</th>
<th>Approx. Size, M (kDa)</th>
<th>Radius ( R_{\text{min}} ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-EGFR antibody</td>
<td>Plasma Membrane</td>
<td>150</td>
<td>3.5</td>
</tr>
<tr>
<td>Anti-Lamp-1 antibody</td>
<td>Lysosome</td>
<td>120</td>
<td>3.3</td>
</tr>
<tr>
<td>Anti-Cox IV antibody</td>
<td>Mitochondrion</td>
<td>17</td>
<td>1.7</td>
</tr>
<tr>
<td>Hoechst dye</td>
<td>Nucleus</td>
<td>0.5</td>
<td>~0.5</td>
</tr>
<tr>
<td>EGF ligand</td>
<td>Plasma Membrane</td>
<td>6</td>
<td>1.2</td>
</tr>
<tr>
<td>EGFR targeted NP</td>
<td>Plasma Membrane</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

The role of silica deposition on the accessibility of molecular and antibody probes with differing dimensions (Figure 2.4B) has also been assessed. Comparatively, it was observed that Hoechst dye (0.5kDa, about 0.5 nm) could still stain the ‘nucleus’ of silicified, dehydrated, and desilicified cells, whereas larger antibody probes were excluded from the dehydrated cells. Based on this observation, I hypothesized that the larger antibody probes could be excluded by
a reduction in the intrinsic pore size of the dehydrated silica mesh. To test this hypothesis, dehydrated silicified cells were labeled with two biomarkers (anti-EGFR antibody and EGF ligand) that have different sizes but almost the same binding site on the EGFR protein. Epidermal growth factor (EGF) ligand at 6kDa and about 1.2-nm in size was able to label/bind EGFR, while the anti-EGFR antibody (150kDa, 3.5-nm) could not (Figure S2.4). The same outcome was observed with anti-Lamp-1 (120 kDa, 3.3 nm) and anti-Cox IV (17 kDa, 1.7 nm) antibodies, which, due to their comparatively larger sizes, were unable to label the respective organelles within the dehydrated silicified cell (Figure 2.4 and Figure 2.5). Together, this preliminary evidence provides a suitable basis to elucidate the preservation of the functions of proteins (e.g. enzymes) and other biomolecular components in silicified cells.
Figure 2.5. Confocal microscopy images of A549 cell plasma membrane and intracellular organelles stained with Anti-EGFR (magenta), Lamp-1 (green), Cox IV (red), and Hoechst (blue) during silicification and desilicification process.

2.2.3 Enzyme activity preservation

Although the integrity of the biomolecular components of the cell together with their intrinsic interactions were preserved during the silicification process, questions still remained as to whether conventional enzyme activities in these biocomposites would be also preserved by silicification. To determine the preservation of enzyme activities in silica biocomposites of HeLa cells, we used commercially sourced probes and biochemical assays. The results showed that esterase, phosphatase (acid and alkali), and protease enzymes in HeLa cell silica biocomposites (with or without dehydration) were just as functionally active as in ‘fixed only’ HeLa cells, suggestive of silicification mediated preservation of the integrity and activity of intracellular and membrane-bound enzymes (Figure 2.3). While esterase, acid phosphatase and protease enzymes are localized in the cell cytoplasm, alkaline phosphatase is primarily localized on the plasma membrane where it plays the crucial role of controlling phosphorylation states of a number of membrane bound proteins. Therefore, by confirming the ability of silicification to preserve functional characteristics of cellular enzymes, we anticipated that this bioinspired approach would facilitate use of silica protected enzymes and other related molecular markers in areas where their stabilities have previously been problematic.
Figure 2.6. A) Fluorescence images of lipid (red) coated silicified HeLa cell showing esterase activity (green) after the silicification and dehydration process. B) Esterase activity of control, fixed cell, fixed and dehydrated cell, fixed and silicified cells, and fixed, silicified and dehydrated cells, respectively. C) Protease activity of control, fixed cell, fixed and dehydrated cell, fixed and silicified cells, and fixed, silicified and dehydrated cells, respectively. D) Acid phosphatase activity of control, fixed cell, fixed and dehydrated cell, fixed and silicified cells, and fixed, silicified, and dehydrated cells, respectively. E) Alkaline phosphatase activity of control, fixed cell, fixed and dehydrated cell, fixed and silicified cells, and fixed, silicified and dehydrated cells, respectively. The error bars indicated the standard deviations of three independent experiments.
To further confirm that silicification can better preserve cellular enzyme activity, we assessed functional activities of enzymes in HeLa cell silica biocomposites subjected to 3 months storage at room temperature. This assessment was premised on two aims: 1) establishing if silicified cells are stable enough to withstand long-term degradation at room temperature instead of commonly used refrigeration or freezing storage conditions, and 2) establishing long-term functional resilience of molecular components in silicified cells. For cells subjected to ‘fixation only’, about 70% acid phosphatase activity and 10% alkali phosphatase activity were still observed after 3-month sample storage at room temperature (Figure 2.4A and 2.4B). This greater retained activity of acid phosphatase activities was attributed to the inherent difference in location between the two enzymes. The alkali phosphatases being mainly localized on the plasma membrane could be susceptible to time-limited and environment driven degradation, while the acid phosphatases that are mainly found in the lysosomes, were perhaps well protected by the lysosomal membrane barrier, which should account for the relatively longer retention of the acid phosphatase activity in the “fixed only” cells. However, for HeLa silica cell biocomposites, both acid and alkali phosphatase enzymes maintained more than 80% activity even after 3 months of storage (Figure 2.4A and 2.4B). Significantly, this finding suggested that, silica cell biocomposites possess elaborate ability to withstand environmental shocks caused by long-term storage at room temperature with no damage to the cellular biomolecules and enzyme activities.
In order to emphasize the resilience of the silica cell biocomposite, we assessed the stability of acid phosphatase and esterase enzymes in HeLa cell silica biocomposites subjected to repeated washes followed by probing of target enzyme activity. The aim was to assess if relative to the ‘fixed only’ sample, the silica biocomposite material was stable and versatile enough to withstand these harsh repeated washing processes that hypothetically, could lead to loss of enzyme activity due to either enzyme leaching from the cell matrix or perturbations of the enzyme structure by the solution system. In the “fixed only” cells, we observed about 40% drop in activity of the esterase enzyme after 6 recycling activity tests relative to the initial enzyme activity test on cycle number 1 (Figure 2.4C). This was contrary to the acid phosphatase enzyme in the “fixed only” cells, which retained over 90% activity regardless of the number of recycling activity tests (Figure 2.4D), further suggesting that the acid phosphatase activity may be protected from degradation by the lysosomal membrane as explained before. However, in the silicified cells, a markedly different phenomenon was observed whereby, both the esterase and acid phosphatase enzyme activities were still maintained at more than 90% even after 6 recycling activity tests (Figure 2.4C and 2.4D). This was attributed to the ability of cell silicification to protect these enzymes from leaching from the cells into the reaction solution which potentially can lead to loss of activity due to denaturation by the solution environment. Together, our findings confirm that, cell silicification ensures requisite protection and stability of various molecular components of the cell.
Figure 2.7. A) Acid phosphatase activity of fixed and dehydrated cell and fixed, silicified and dehydrated cells stored 1 week and 3 months, respectively. B) Alkaline phosphatase activity of fixed cell, fixed and dehydrated cell, fixed and silicified cells, and fixed, silicified and dehydrated cells stored 1 week and 3 months, respectively. C) Recycling stability of acid phosphatase in fixed cell and fixed, silicified and dehydrated cells for six times. D) Recycling stability of esterase in fixed cell and fixed, silicified and dehydrated cells for six times. The relative activity was calculated using the residual activity at each time point relative to that found initially on cycling number 1.

2.3 Conclusion

Dehydrated silicified cells, which we refer to as hybrid silica biocomposites, possess unique and important features such as ability to withstand long-term
storage at room temperature with no damage to the cellular biomarkers and enzyme activities. We have shown that we can use antibody and nanoparticle probes to determine the accessibility and preservation of these biomarkers displayed on both the extracellular membrane and intracellular compartments throughout the course of silicification, dehydration, and desilicification. We find that molecular and antibody probes ranging in size from 0.5 to 3.5-nm in diameter bind to extracellular membranes and intracellular compartments in a manner similar to fixed cells but that all but the smallest probe (Hoechst dye $R_{\text{min}} = 0.5$-nm) are excluded from the dehydrated silicified cells. Etching of about 12-nm of silica restores accessibility of all probes in a manner similar to fixed cells. These finding suggest that silicification followed by drying can preserve and passivate biofunctional features within a protective silica coating and that exposure to a mild etchant could re-activate biofunctions. We have also shown that the activities of esterase, phosphatase (acid and alkali), and protease enzymes in hybrid silica biocomposites are preserved both in the short and long-term. Here we might expect the preserved 3D intracellular scaffold to provide a naturally crowded microenvironment for enhancing biofunctionality.
2.4 Materials and Methods

2.4.1 Materials

All chemicals and reagents were used as received. Heat inactivated fetal bovine serum (FBS), 10X phosphate-buffered saline (PBS), 1X trypsin-EDTA solution, and penicillin streptomycin (PS) were purchased from Gibco (Logan, UT). Dulbecco’s modification of Eagle's medium with 4.5g/L glucose, l-glutamine, and sodium pyruvate (DMEM) and F12-K medium were obtained from CORNING cellgro (Manassas, VA). Formaldehyde solution (36.5-38% in H₂O) and Tetramethyl orthosilicate (TMOS) was purchased from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid (36.5–38%, HCl) was purchased from EMD Chemicals (Gibbstown, NJ). Absolute (200 proof) ethanol were obtained from PHARMCO-AAPER (Brookfield, CT). Anti-Cox IV antibody was purchased from Cell Signaling Technology (British English). Anti-Lamp-1 antibody and anti-EGFR antibody were purchased from Santa Cruz Biotechnology (Dallas, TX). CM-H2DCFDA, EnzChek™ Protease Assay Kit, and 4-Methylumbelliferyl Phosphate (MUP) were purchased from Thermo Scientific (Waltham, MA). p-Nitrophenyl Phosphate (PNPP) was purchased from New England Biolabs (Ipswich, MA).

2.4.2 Cell culture and chemical fixation.

Cell culture was performed using standard procedures (atcc.org). Briefly, A549 (CCL-185) and Hela (CCL-2) were obtained from American Type Culture Collection (ATCC) and maintained in media containing 10% FBS at 37°C and 5% CO₂. Cells were passaged at approximately 80% confluency. For imaging
purposes, 100,000 cells/mL A549 cells were seeded on glass substrates and
cultured 24h. After 24h, cells were fixed in 4% formaldehyde (in 1X PBS) at room
temperature for 15 min, rinsed with 1X PBS and stored in 1X PBS at 4°C. For
enzymatic activity study, HeLa cells were removed from plate bottom using
Trypsin-EDTA (0.25%).

2.4.3 Silicification and desilicification of mammalian cells.

Fixed mammalian cells were immersed in a solution containing 0.1 M TMOS
in 1.0 mM HCl (pH 3) and rotated 24 hours at room temperature for silicification.
For hydrated samples, silicified cells were rinsed with deionized water followed by
rinsing in 1X PBS, and stored in 1X PBS at 4°C. For dehydration, silicified cells
were dehydrated by sequential rinsing in deionized water, 30% ethanol, 50%
ethanol, 70% ethanol, 90% ethanol, 100% ethanol for 10 min in each solution and
then dried under vacuum. Dehydrated samples are stored at room temperature.
Before using, dehydrated samples were rehydrated by immersing in 1X PBS for
24hours. Rehydrated samples were rinsed in 1X PBS and stored in 1X PBS for
following measurements. In order to do the desilicification, buffered oxide etch,
also known as buffered HF (BOE) solution (pH~5) was used to etch the silica and
yield desilicified cells. [Caution! HF is highly toxic. Extreme care should be taken
when handling HF solution and only small quantities should be prepared.] The
desilicified cells were washed with water and stored in 1X PBS for following
measurements.
2.4.4 Immunofluorescence staining

The samples were blocked with 5% BSA in 1x PBS, and then incubated with fluorescent antibodies against Cox IV, Lamp-1 and EGFR proteins for 30min. The samples were then rinsed with 1x PBS, and then stored in 1x PBS. Microscopy images were then obtained on the Leica DMI3000 B inverted fluorescence microscope or Leica TCS SP8 confocal microscope.

2.4.5 Enzymatic activity study

The enzymatic activity assay were followed the standard protocol provided with enzyme substrate. The absorbance or fluorescence of signal was measured using a BioTek microplate reader (Winooski, VT).
2.5 References


CHAPTER 3

SupraCells: Living Mammalian Cells Protected within Functional Modular Nanoparticle-Based Exoskeletons

This chapter was adapted from
Wei Zhu‡, Jimin Guo‡, Shahrouz Amini, Yi Ju, Jacob Ongudi Agola, Andreas Zimpel, Jin Shang, Achraf Noureddine, Frank Caruso, Stefan Wuttke, Jonas G. Croissant*, C. Jeffrey Brinker* SupraCells: Living Mammalian Cells Protected within Functional Modular Nanoparticle-Based Exoskeletons. Submitted to Advanced Materials. (‡Authors contributed equally to this work)
3.1 Introduction

Enhancing or augmenting the performance of mammalian cells could result in new classes of smart responsive living materials. Mammalian cells exhibit complex functionalities like sensing signal transduction and protein expression but they remain fragile and highly susceptible to intracellular and extracellular stressors.[1-4] Recently, to impart greater cellular durability, cytoprotective material nanolayers, such as silica, titania, cross-linked polymer and metal-phenolic networks, have been coated on individual mammalian cells, increasing resistance against UV, freezing, and enzymatic attack.[5-8] However, the current cell encapsulation approaches possess a number of limitations, including the poor control of permeability, multiple processing steps, the lack of versatile capability to endow cells with multiple functionalities for biomedical applications, and the bio-incompatibility of most material synthesis conditions (pH, temperature, precursor concentration, etc.) with limiting cellular survival.[9-14] These limitations necessitate a new cytoprotective encapsulation strategy.

Synthetic nanoparticles (NPs) with various chemical compositions and diverse functionalities naturally interact with mammalian cell surfaces through multiple non-covalent binding interactions developed with proteins and other cellular membrane components.[15-17] Often these interactions lead to NP accumulation and subsequent internalization by phagocytosis or macropinocytosis based on membrane extension or invagination and wrapping of individual or groups of nanoparticles.[18-20] The natural coherence of the NP/cellular membrane interface suggests that NPs might be ideal candidates for cellular encapsulation if
accompanying NP internalization mechanisms could be suppressed. Here, we describe a general means of uniform cellular encapsulation within a NP-based exoskeleton employing arbitrary NP building blocks (and combinations thereof) and inter-nanoparticle ligands (Figure 3.1A). This simple universal encapsulation approach, referred to here as ‘SupraCells’, instantly encapsulates living mammalian cells within functional, modular, NP-based exoskeletons avoiding typical endocytotic nanoparticle internalization pathways. It provides an ability to endow the encapsulated cell with useful, tunable physico-chemical properties (e.g., optical, magnetic, and sensing properties) depending on the NPs or NP combinations (Figure 3.1B). The potential chemical diversity of SupraCells is enormous, and here we demonstrated SupraCell prototypes prepared with NP-exoskeletons including metal-organic frameworks (MOFs) (i.e., ZIF-8, MIL-100, UiO-66-NH₂, MET-3-Fe types, vide infra), mesoporous silica NPs (mSiO₂ and dye-labeled mSiO₂), iron oxide (Fe₃O₄) NPs (Figure 3.1B) and NP combinations. SupraCell formation maintains normal cellular functions (e.g., viability, metabolism) but induces a spore-like state, wherein cells do not replicate or spread on surfaces but are endowed with extremophile properties, e.g., resistance to osmotic stress, reactive oxygen species (ROS), pH, and UV exposure. NP functionality confers to the cell abiotic properties including tunable cell-mechanics, selective permeability, intracellular activity sensing, multi-fluorescence, magnetism, and conductivity, which are utterly foreign to the native mammalian cells.
3.2 Results and Discussion

3.2.1 Formation and characterization of SupraCells

As a first demonstration of the SupraCell concept, individual HeLa cells encapsulated within ZIF-8 (zeolitic imidazolate framework-8) NP-based exoskeletons (termed Supra-HeLa Cell-ZIF-8) were constructed via the sequential addition of a colloidal ZIF-8 solution and tannic acid to cell suspensions prepared in PBS solution (see materials and methods in SI for detailed information). The ca. 50 nm diameter, well-defined rhombic dodecahedral shape, and cubic \( I \bar{4} 3 \) m group symmetry of water borne colloidal ZIF-8 NPs were confirmed using transmission electron microscopy (TEM) and wide-angle X-ray diffraction (XRD) analyses (Figure 3.1D,F). Only thirty seconds of incubation were necessary to freeze the cellular internalization of the ZIF-8 nanobuilding blocks via tannic acid-mediated interparticle binding due to strong-multivalent metal-phenolic complexation.[21-22]

The formation of the NP-based exoskeleton surrounding the HeLa cells is driven by the multitude of NP-cell membrane interactions. We directly visualized the NP exoskeleton using bright field and scanning electron microscopy (SEM) imaging of both normal cells and SupraCells (Figure 3.1C,E; Figure S3.1). Fourier-transform infrared spectroscopy performed on Supra-HeLa Cell-ZIF-8 confirmed the coordination of tannic acid to zinc open sites on the ZIF-8 surface, as evidenced by the characteristic peaks at 1179 and 994 cm\(^{-1}\) assigned to the vibration of C=N and C−N in the imidazole ring of ZIF-8 and 1083 cm\(^{-1}\) assigned to the stretching vibration of C-O in tannic acid (Figure S3.2), respectively. Analyzing nearly one
hundred SupraCells on bright field and SEM images strongly supported the fact that all individual HeLa cells had homogeneous conformal exoskeletons (Figure 3.1G; Figure S3.3), as further confirmed by confocal scanning laser microscopy (CLSM) of a rhodamine-labeled ZIF-8-NP-based exoskeleton (Figure 3.1H), where we observe a coherent, conformal ZIF-8-NP layer encapsulating the HeLa cell.
Figure 3.1. Formation and characterization of SupraCells. (A) Schematic representation of SupraCell formation via immediate, self-limiting ligand assisted formation of NP exoskeletons that ‘freeze’ natural mammalian endocytotic pathways. (B) Representation of various NP building blocks including MOFs (ZIF-8, MIL-100, UiO-66, MET-3-Fe), mesoporous silica (mSiO$_2$), and iron oxide (Fe$_3$O$_4$) NPs. (C) Bright field (left) and scanning electron (right) images of HeLa cells. (D) Transmission electron image of ZIF-8 nanobuilding blocks. (E) Bright field (left) and scanning electron (right) images of HeLa SupraCells based on ZIF-8 nanobuilding blocks and tannic acid as inter-particle ligands. (F) X-ray diffraction pattern of Supra-HeLa Cell-ZIF-8 and ZIF-8 NPs. (G) Low-magnification bright field image of Supra-HeLa Cell-ZIF-8. (H) Z-stack confocal image of a SupraCell demonstrating the homogeneous formation of the NP-based exoskeleton (red-colored from rhodamine labeled ZIF-8 NPs).

Wide-angle XRD (Figure 3.1F) along with energy-dispersive X-ray (EDX) spectroscopy mapping of zinc, carbon, and oxygen atoms (Figure S3.4) confirmed preservation of the structural and chemical integrity of the ZIF-8-NP exoskeletons.\[^{23-24}\] The generality of the NP-based exoskeleton paradigm was then demonstrated on other mammalian cell lines including A549 cells (adenocarcinomic human alveolar basal epithelial cell) and HL-60 cells (human promyelocytic leukemia cells), both yielding SupraCells-ZIF-8 with continuous exoskeletons (Figure S3.5-3.6).

We then sought to demonstrate the versatility of the SupraCell approach by extending it to different nanobuilding blocks for multifunetion integration. Additional types of MOF NPs (e.g. MIL-100(Fe), UiO-66-NH$_2$, MET-3-Fe) with different framework-related functionalities (e.g. sensing, and conductivity), mSiO$_2$ and dye-labeled mSiO$_2$, as well as magnetic Fe$_3$O$_4$ NPs were selected for SupraCell exoskeleton formation experiments employing HeLa cells. For every case, successful preparation of NP was confirmed by a panel of analyses including XRD, SEM, TEM, and dynamic light scattering (DLS) (Figure S3.7-3.11). For the different NP systems, we used different inter-particle ligand chemistries to form the
exoskeletons via inter-nanoparticle binding at the cellular interface, namely, tannic acid for MOF systems based on metal-phenolic interactions,\textsuperscript{[21-22]} 1,4-benzendiboronic acid for phenol-functionalized mSiO\textsubscript{2} or Fe\textsubscript{3}O\textsubscript{4},\textsuperscript{[25]} exploiting boronate-phenolic interactions, and 4-arm-PEG5K-SH for thiol-modified mSiO\textsubscript{2} through thiol-thiol reactions\textsuperscript{[26]} (Figure S3.12). We then successfully extended these SupraCell syntheses to different cell lines (e.g., Raw 264.7 cell lines, Scheme S3.1). Based on characterization by SEM, optical microscopy, and CLSM, all SupraCell constructs depicted continuous, conformal NP-based exoskeletons (Figure S3.13-3.16). As a control without inter-nanoparticle crosslinking, the NPs were quickly (< 5 min) physically adsorbed onto the cellular surface and then internalized by the cell and accumulated around the nucleus (Figure S3.17). However, after the construction of NP-based exoskeletons, the NPs internalization could be further blocked (Figure S3.18). The robustness and versatility of our approach using various mammalian cell lines and nanobuilding blocks hence strongly suggests that a vast library of SupraCells can now be designed for a wide array of scientific investigations.

Implicit in the SupraCell concept of protecting cells within NP exoskeletons is preservation of cellular function. In order to assess the cytocompatibility of the SupraCell process, we determined the viabilities of HeLa-, A549-, Raw 264.7-, and HL-60-based SupraCell suspensions using the CellTiter-Glo\textsuperscript{®} 2.0 cell viability kit. All the SupraCells exhibited cell viabilities of at least 90% (Figure S3.19), indicating negligible cytotoxicity of the exoskeleton formation process. Extending the incubation times up to 72 h reduced the viability in an identical manner to that
of native HeLa cells maintained in suspension (Figure 3.2A). Here it should be noted that normally adherent cells maintained in suspension, which lose their cell–extracellular matrix (ECM) interactions, undergo a process of anoikis where the cell cycle is arrested and a specific form of caspase-mediated programmed cell death (apoptosis) occurs.[27]

Having established Supracell viabilities, we next examined the related biological behaviors. We hypothesized that the expected rigidity (vide infra) of the NP exoskeleton, cellular isolation, and obscuration of cell adhesion molecules like integrins would conspire to arrest cellular adhesion, spreading, and proliferation. To test this hypothesis, we evaluated the proliferation of HeLa based SupraCell-ZIF-8 suspensions when introduced to glass substrates under standard culture conditions at time points ranging from 1-24 h post exoskeleton formation and compared it to that of native HeLa cells. As shown in Figure S3.20, unlike native HeLa cells, SupraCells do not adhere, spread or proliferate. However, based on the reversibility of metal-phenolic complexation, exposure of SupraCell-ZIF-8 to ethylenediaminetetraacetic acid solution (EDTA, 20 mM, pH 5.0) for 30 min results in Zn chelation, complete exoskeleton removal, and recovery of native HeLa cell behavior. Figure S3.21 shows the formation and removal of ZIF-8 or MIL-100(Fe) exoskeletons had a negligible effect on viability compared to native control cells. As shown in Figure S3.22, after the removal of exoskeleton, HeLa cells can again adhere, spread, and proliferate under cell culture conditions. Analysis of proliferation rates indicate that the ‘reversed’ SupraCells have almost the same proliferation rate compared to native cells (Figure S3.23-3.24). This on-demand
exoskeleton formation and degradation capability confers to the mammalian cells behaviors normally associated with the germination of natural spores.

3.2.2 Enhanced resistance of SupraCells against endo- and exogenous stimuli

![Graphs showing viability of cells under various conditions.]

**Figure 3.2. Enhanced resistance of SupraCells against endo- and exogenous stimuli.** (A) Viability of HeLa cells versus corresponding SupraCells based on MIL-100(Fe) nanobuilding blocks, in normal conditions. (B) Viability of HeLa cells versus SupraCells-MIL-100(Fe) as a function of the salt concentration (i.e. osmotic pressure stimulus), (C) H₂O₂ concentration (i.e. ROS stimulus), (D) pH, (E) UV irradiation time (254 nm), and (F) toxic silver NPs (diameter: ~5.0 nm).

In order to demonstrate how the SupraCell exoskeletons protect the cells against external stressors, SupraCell-MIL-100(Fe) was exposed to various osmotic pressure, pH, reactive oxygen species (ROS), UV stressors, and toxic NPs (**Figure 3.2**). First upon exposure to varying ionic strength solutions
(expressed as x PBS, where 1x PBS is isotonic with living cells), native HeLa cells show 100% viability at 1x PBS but greatly reduced viability at lower or higher osmotic stress and only 6.4% and 26.0% viability at 0.25x PBS and 5x PBS, respectively. In comparison SupraCell-MIL-100(Fe) shows nearly 100% viability from 0.75x PBS to 3x PBS and cell viabilities of 31.0% and 44.7%, at 0.25x PBS and 5x PBS, respectively. In mammalian cells, hypertonic conditions result in water expulsion from cells and cell shrinkage, while hypotonic conditions result in cell swelling, both processes resulting in rapid cell lysis for conditions other than isotonic. The enhanced mechanical stiffness and membrane reinforcement provided by the SupraCell exoskeleton (vide infra) resists both cellular shrinkage and swelling processes, as shown in Figure S3.25, thereby greatly reducing cell lysis under hypotonic and hypertonic conditions.

Second, ROS can cause oxidative damage and produce adverse modification to cellular components (e.g. lipids, and DNA). As shown in Figure 3.2C, the viabilities of SupraCells paralleled but were statistically greater than those of native HeLa cells in the presence of increasing hydrogen peroxide (H₂O₂) concentrations. The increased ROS-resistance of SupraCells may be associated with the unique antioxidant properties of tannic acid in the exoskeleton nanostructure. Third, the viability of SupraCells was tested over the pH range 4-11 as it is known that altered acid-base balance and extreme pH ranges can disrupt cell metabolic processes and cause irreversible cell damage. As shown in Figure 3.2D, pH values below 6.0 or greater than 8.0 led to an abrupt decrease of viability for native HeLa cells, whereas SupraCells showed increased resistance toward pH variation and
exhibited two-fold and three-fold higher viabilities at pH 11 and 4, respectively. We attribute this pH resistance to the ion chelating effect resulting from the porous exoskeleton framework.[31-33] Fourth, we determined the resistance of SupraCells to UV irradiation (\( \lambda = 254 \text{ nm}, 4W \)) in comparison to native mammalian cells. As expected, UV exposure caused a sharp decline in the survival of native cells after 60 min, resulting in about 30% survival,[34] whereas SupraCells remained largely unaffected after the same exposure time (Figure 3.2E and Figure S3.26). After two hours of UV exposure, the viability of SupraCells was over six times higher than the native cells. We attribute the UV resistance to the high UV-absorption coefficient of the MIL-100(Fe)-based SupraCell exoskeletons over the wavelength range 200–300 nm (Figure S3.27). Furthermore, the intrinsic porosity of MIL-100(Fe) enables loading of UV-absorbing dyes (e.g. Congo red) in the exoskeleton further enhancing the protection of SupraCell-MIL-100 against UV irradiation (Congo red-loaded SupraCell-MIL-100(Fe) SupraCells exhibited 15% greater viability upon exposure to 365 nm UV light for 120 min compared to unloaded SupraCells) (Figure S3.28). The ability to load molecular cargos into the MOF exoskeleton opens vast possibilities to tune the properties of SupraCells. Finally, both native cells and SupraCells were exposed to cytotoxic silver (Ag) nanoparticles (diameter: \( \sim 5.0 \text{ nm} \)) at different concentrations. As shown in Figure 3.2F, native cells were very sensitive to toxic Ag NPs and showed a dose-dependent response behavior. On the contrary, Supracells exhibited \( \sim 100\% \) viability even at high concentrations of 12 \( \mu g/mL \), highlighting the excellent protection conferred by the NP-based exoskeletons.
3.2.3 Enhanced mechanical resistance of SupraCells

The mechanical behavior of MOF-based SupraCells was determined by nanoindentation and compared to native cells (Figure 3.3A). HeLa-based SupraCell-ZIF-8 and SupraCell-MIL-100(Fe) samples along with native HeLa cells were subjected to multiple loading-unloading cycles to differing contact depths (Figure S3.29). Due to the size and softness of the cell samples, a Berkovich tip with wide contact angle (142.3°) was used. Extracted loading-unloading curves (Figure S3.29) revealed that the contact depths for the SupraCells were ~50% lower than the contact depths of native HeLa cells, indicating the higher stiffness afforded by the exoskeletons. Slopes of the loading-unloading curves were then used to determine the stiffness $S$ and elastic modulus $E$ as a function of contact depth (Figure S3.30). Figure 3.3B compares $S$ and $E$ for SupraCells and native HeLa cells indented to a contact depth of 100-nm (the approximate thickness of the exoskeleton) where we find SupraCells to have 2-4 times greater stiffness and modulus, explaining in part their greater resistance to osmotic stress. ZIF-8 and MIL-100(Fe)-based SupraCells were also found to have differing contact depth dependencies of their elastic moduli (Figure S3.30), whereby, MIL-100(Fe) is a flexible MOF that can exhibit densification under applied pressure while ZIF-8-based SupraCells are not based on flexible nanostructures.$^{35-36}$

As represented in Figure 3.4, the SupraCell construct can be extended to a large number of nanoparticle types and combinations to achieve varied functions. A common function of all SupraCells is that the uniform and complete encapsulation with various NPs introduces a non-native, size-selective
permeability to the exoskeleton that can maintain viability while serving to protect
the cell against certain exogenous toxins and pathogens. To demonstrate the
aspects of size selective permeability, SupraCell-MIL-100(Fe) was selected
because, as shown in Figure S3.31, it is composed of two connected mesocage
networks that act as molecular sieves preventing transport of entities greater than
2.9 nm in kinetic diameter. First, preservation of the permeability of the Supracell
to molecular components was demonstrated using labeled glucose tracers (2-
Deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose (2-NBDG)) (Figure
3.4B).

Figure 3.3. Enhanced mechanical resistance of SupraCells. (A) Representation of the
mechanical response test set-up involving the Berkovich Intender imposing a P load onto cells
(left) and SupraCells (right) using a cyclic loading-unloading function. This cyclic load function
has been selected to ensure that the measurements have not been affected by the surface
roughness. (B) Stiffness and elastic modulus of HeLa cells and SupraCells-MIL-100(Fe) or
ZIF-8 (M or Z refers to MIL-100(Fe) or ZIF-8, respectively; the number refers to the coating
cycle).
Then, the anticancer drug doxorubicin (diameter ~1.6 nm) was added into the culture medium at different concentrations. As shown in Figure S3.32, both native cells and SupraCells were sensitive to doxorubicin and each exhibited a nearly identical dose response curve. Combined with the described results in Figure 3.2F where native cells but not SupraCells were sensitive to Ag NPs (diameter ~5.0 nm), these results established an effective pore size cut-off of the exoskeleton membrane (> 5 nm) consistent with the pore size of the MIL-100(Fe). The MIL-100(Fe)-based exoskeleton also prevented phagocytosis of GFP-expressing Salmonella enterica serovar Typhimurium LT2 bacteria (Figure 3.4C). SupraCells are thus endowed with a unique semi-permeability allowing nutrient uptake but denying attack by pathogens. As depicted in Figure 3.4A the SupraCell concept can be extended generally to other NP types and combinations to achieve diverse functionalities including multifluorescent labeling, sensing, magnetic, and conductive properties (Figure 3.4D-G), while maintaining >90% viability of all respective SupraCells (Figure S3.18). As an example, multi-fluorescent SupraCells were fabricated by incubating HeLa cells simultaneously with equal concentrations of three different fluorescently labeled mesoporous silica nanoparticles in a one-pot process for less than one minute (Figure S3.33). Confocal microscopy images in 2D and 3D demonstrated the formation of a continuous exoskeleton and a homogeneous distribution of MSNs that preserved the stoichiometry of the synthesis solution (Figure 3.4E). We next designed sensing SupraCells to demonstrate in-situ monitoring of intracellular nitric oxide (NO), which is a key signaling molecule in many pathological processes.\[37] NO
sensing was achieved using luminescent MOF nanobuilding blocks (UiO-66-NH₂ NPs) whose luminescence is quenched upon NO-triggered de-amination, allowing real time NO detection.[38] Using metal-phenolic linker chemistry, Mouse macrophage Raw 264.7 cells were encapsulated with UiO-66-NH₂ NP-based exoskeletons as demonstrated by the blue fluorescent coronas observed around the surface of SupraCells (Figure S3.34).

3.2.4 SupraCell-properties based on modular functional NP-based exoskeletons

SupraCells were then exposed to lipopolysaccharide (LPS), a traditional exogenous activator of the TLR4 (toll-like receptor 4)-NF-κB-iNOS (inducible nitric oxide synthase) (Figure S3.35) pathway[39] resulting in time-dependent luminescence quenching indicative of NO detection (Figure 3.4D). After 32 h incubation, UiO-66-NH₂ SupraCells exposed to LPS exhibited ~25% quenching relative to SupraCells not exposed to LPS that showed no significant quenching. Magnetic SupraCells that hold great potential as magnetic resonance imaging (MRI) agents and micro-motorized cellular constructs were prepared using ca. 8.0-nm diameter iron oxide (Fe₃O₄) nanobuilding blocks.[40] Using boronate-phenolic linker chemistry, HeLa cells were encapsulated within a continuous magnetic Fe₃O₄ exoskeleton (Figure S3.16), which allows the movement of cells to be controlled via an external magnetic field (Figure 3.4F).
Figure 3.4. SupraCell-properties based on modular functional NP-based exoskeletons.

(A) Representation of various SupraCells characterized by one or more nanobuilding block type. (B-C) Size-selective permeability studies of SupraCell-MIL-100(Fe) involving (B) sugar permeation and (C) bacteria non-permeation (confocal images show the intracellular green fluorescence of bacteria only in normal cells). (D) Timeline of the fluorescence of cellular NO-sensing SupraCell based on fluorescent UiO-66(Ph-NH$_2$) nanobuilding blocks upon NO detection. (E) Confocal images of multi-fluorescent SupraCell based on three different
fluorescent mSiO$_2$ nanobuilding (mSiO$_2$-1-2-3) blocks. (F) Bright-field microscopy images of magnetically-actuated SupraCell-Fe$_3$O$_4$ (left) or immobile normal cell (top right). Photographs of a dispersion of SupraCells before (left) and after (right) placing a magnet on its side. (G) Current-voltage plot demonstrating the conductivity imparted to SupraCells via MET-3 (Fe) MOF NP-based exoskeletons.

Finally electrically conductive SupraCells were synthesized using electrically conductive metal-triazolate MOF of MET-3(Fe) NPs.$^{[41]}$ As shown in Figure S3.13, HeLa cells were uniformly encapsulated within MET-3(Fe) exoskeletons. The ohmic conductivity of the SupraCell-MET-3(Fe) was assessed via in-situ SEM electrical characterizations (Figure 3.4G and Figure S3.36). By placing the SEM probe onto the SupraCell surfaces, representative current-voltage (IV) curves for SupraCell-MRT-3(Fe) and HeLa cells were reliably obtained (Figure 3.4G). A high resistivity (~8.75 MΩ) was measured for the native cells, as expected from the non-conductive cell cytoskeletons and intracellular components. In contrast, a dramatic decrease in the resistance by approximately 3600-fold (~2.4 kΩ) was measured for the SupraCell-MRT-3(Fe). Conductive SupraCells thus appear as promising living microdevices for applications in biological fuel cells.

3.3 Conclusion

We have developed a general, simple, and modular approach to create a new class of living hybrid materials termed SupraCells with diverse possible functionalities. Using linker chemistries we encapsulate mammalian cells within nanoparticle-based exoskeletons in an instantaneous process that avoids/abolishes NP internalization pathways such as phagocytosis. The NP
exoskeletons are shown to be continuous and to maintain cell viabilities for a long period of time in a non-replicative state endowed with extremophile-like properties. Metal chelation disrupts the linker chemistry and cells return to their native states. The exoskeletons exhibit size selective permeability protecting the cells against toxins and pathogens exceeding 5-nm in diameter. Potential SupraCell functionalities are as diverse as the NP exoskeleton building blocks themselves. SupraCells demonstrated high viabilities with preserved typical metabolic signatures of the native cells, enhanced resistances against both endogenous and exogenous stressors, and extraordinary properties foreign to native cells based on the nature of the nanobuilding blocks integrated into their unique exoskeletons. We envision that this modular coating strategy will open new avenues to simple manufacturing pathways of complex micro- and/or macroscale hybrid mammalian cell-based functional materials and devices.
3.4 Materials and Methods

3.4.1 General information

**Reagents.** All chemicals and reagents were used as received. Zinc nitrate hexahydrate, 2-methylimidazole, zirconium(IV) chloride, terephthalic acid, 2-aminoterephthalic acid, dimethylformamide (DMF), trimesic acid, iron(III) chloride hexahydrate, tetraethyl orthosilicate (TEOS), (3-aminopropyl)triethoxysilane (APTES), ammonium hydroxide, ammonium nitrate, hexadecyltrimethylammonium bromide (CTAB), cyclohexane, tannic acid, benzene-1,4-diboronic acid, ethylenediaminetetraacetic acid, rhodamine B isothiocyanate mixed isomers, fluorescein isothiocyanate, iron(III) acetylacetonate (Fe(acac)₃), copper(II) nitrate, 7,7,8,8-tetracyanoquinodimethane (TCNQ), benzyl alcohol, methanol, Ham's F-12K (Kaighn's) medium, Iscove's modified Dulbecco's media (IMDM), formaldehyde solution (36.5-38% in H₂O), dimethyl sulfoxide (DMSO), doxorubicin (DOX), silver nanoparticles, Congo red, 5,10,15,20-tetrakis(4-sulfonatophenyl)-21H,23H-porphine manganese (III) chloride, and gentamicin were purchased from Sigma-Aldrich. 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-deoxyglucose (2-NBDG), Alexa Fluor™ 633 NHS ester (succinimidyl ester) were purchased from Thermo Fisher Scientific. Heat-inactivated fetal bovine serum (FBS), 10X phosphate-buffered saline (PBS), 1X trypsin-EDTA solution, and penicillin-streptomycin (PS) were purchased from Gibco (Logan, UT). Dulbecco’s modification of Eagle’s medium (DMEM) was obtained from Corning Cellgro (Manassas, VA). Absolute (200 proof) ethanol was obtained from Pharmco-Aaper (Brookfield, CT). CellTiter-Glo 2.0 Assay was purchased from Promega (Madison, WI). Hoechst 33342 were obtained from Thermo Fisher Scientific (Rockford, IL). 1X phosphate-buffered saline (PBS), Alexa Fluor 488 phalloidin and rhodamine phalloidin were purchased from Life Technologies (Eugene, OR). Milli-Q water with a resistivity of 18.2 MΩ cm was obtained from an inline Millipore RiOs/Origin water purification system.

**Characterization.** Scanning electron microscopy (SEM) analyses and
energy-dispersive X-ray spectroscopy (EDS) elemental mappings were performed on a Hitachi SU-8010 field-emission scanning electron microscope at 15.0 kV. Transmission electron microscopy (TEM) and high-resolution TEM (HRTEM) imaging were carried out using a Hitachi model H-7650 transmission electron microscope at 200 kV. Wide-angle powder X-ray diffraction (PXRD) patterns were acquired on a Rigaku D/MAX-RB (12 kW) diffractometer with monochromatized Cu Kα radiation (λ = 0.15418 nm), operating at 40 kV and 120 mA. The UV-Vis absorption spectra were recorded using a Perkin-Elmer UV/vis Lambda 35 spectrometer. The fluorescence emission measurements were carried out using a fluorescence spectrometer (Perkin-Elmer LS55). To characterize the mechanical properties of the samples, a Triboindenter TI950 (Bruker-Hysitron) equipped with a standard 2D transducer and Berkovich tip were used. Three-color images were acquired using a Zeiss LSM510 META (Carl Zeiss MicroImaging, Inc.; Thornwood, NY, USA) operated in channel mode of the LSM510 software.

### 3.4.2 Nanoparticles synthesis

**ZIF-8 NPs synthesis.** ZIF-8 NPs were synthesized following previously reported methods with minor modification. First, 2.27 g 2-methylimidazole was dissolved in 8.0 g Milli-Q water, and then 0.117 g Zn(NO$_3$)$_2$$\cdot$6H$_2$O dissolved in 0.8 g Milli-Q water was added under fast stirring (6000 rpm). The operation was performed at room temperature. After stirring for 15 min, the particles were collected by centrifuging, and then washed with ethanol several times. The synthesized ZIF-8 NPs were stored in EtOH before use.

**MIL-100(Fe) NPs synthesis.** MIL-100(Fe) NPs were synthesized following previously reported methods with no modification. Briefly, 2.43 g iron(III) chloride hexahydrate (9.0 mmol) and 0.84 g trimesic acid (4.0 mmol) in 30 ml H$_2$O were mixed in a Teflon tube, sealed, and placed in the microwave reactor (Microwave, Synthos, Anton Paar). The temperature of the mixed solution was fast increased to 130 °C under solvothermal conditions (P = 2.5 bar) within 30 seconds, and then kept at 130 °C for 4 minutes and 30 seconds, and finally cooled down again to
room temperature. The synthesized NPs were centrifuged down and then washed twice with EtOH. The dispersed NPs were allowed to sediment overnight, and then the supernatant of the sedimented suspension was filtrated (filter discs grade: 391, Sartorius Stedim Biotech) three times to finally yield the required MIL-100(Fe) NPs. The synthesized MIL-100(Fe) NPs were stored in EtOH before use.

**UiO-66/UiO66-NH2 NPs synthesis.** Uio-66 NPs were synthesized following previously reported methods with no modification. Briefly, 25.78 mg ZrCl4 (0.11 mmol) and 13.29 mg 1,4-benzenedicarboxylic acid (0.08 mmol) were dissolved in 10 mL of DMF solution. Then 1.441 g acetic acid (0.024 M) was added into the above solution. The mixed solution was placed in an oven (120 °C) for 24 h. After the reaction mixture was cooled to room temperature, the resulted NPs were subsequently washed with DMF and methanol via centrifugation redispersion cycles. The synthesized Uio-66 NPs were stored in EtOH before use. For the synthesis of UiO66-NH2, the same protocol was used except the replacing the organic ligand 1,4-benzenedicarboxylic acid to 2-amino terephthalic acid.

**MET-3 (Fe) NPs Synthesis.** MET-3 (Fe) NPs were synthesized following previously reported methods with no modification. Briefly, 1.22 g Cu(NO3)2•3H2O (5.24 mmol) and 0.58 g trimesic acid (2.76 mmol) were first dissolved in 5 g DMSO solution to form the precursor solution. Then 0.2 mL of the precursor solution was dropped into 10 mL methanol solution under stirring in 1 min. After the stirring was continued for 20 min, the precipitate was collected by centrifugation and washed several times with methanol. The synthesized HKUST-1 NPs were stored in MeOH before use.

**Mesoporous silica NPs (mSiO2) synthesis.** MSN NPs were synthesized following previously reported methods in our group with no modification. Briefly, 0.29 g of CTAB (0.79 mmol) was dissolved in 150 mL of 0.51 M ammonium hydroxide solution in a 250 mL beaker, sealed with parafilm (Neenah, WI), and placed in a mineral oil bath at 50 °C. After continuously stirring for 1 h, 3 mL of 0.88 M TEOS solution in EtOH and 1.5 μL APTES were combined and added
immediately to the mixed solution. After another 1 h of continuous stirring, the particle solution was stored at 50 °C for another 18 h under static conditions. Next, the solution was passed through a 1.0 μm Acrodisc 25 mm syringe filter (PALL Life Sciences, Ann Arbor, MI) followed by a hydrothermal treatment at 70 °C for 24 h. To remove the CTAB, the synthesized mSiO₂ NPs were transferred to 75 mM ammonium nitrate solution in ethanol, and placed in an oil bath at 60 °C for 1 h with reflux and stirring. The mSiO₂ NPs were then washed in 95% ethanol and transferred to 12 mM HCl ethanolic solution and heated at 60 °C for 2 h with reflux and stirring. Finally, mSiO₂ NPs were washed in 95% ethanol, then 99.5% ethanol, and stored in 99.5% ethanol before use.

**Fe₃O₄ NPs synthesis.** Bare Fe₃O₄ NPs were synthesized following the reported methods with no modification.² Briefly, 0.687 g of Fe(acac)₃ (1.94 mmol) was dissolved in 9 mL of benzyl alcohol. The mixed solution was heated to 170 °C with reflux and stirring at 1500 rpm for 24 h. After the reaction was cooled down to room temperature, 35 mL EtOH was added into the mixed, and then centrifuged at 20000 rpm for 10 min. The supernatant was discarded, and the resulted precipitate was washed with EtOH twice to yield to the required Fe₃O₄ NPs. The synthesized Fe₃O₄ NPs were stored in EtOH before use.

### 3.4.3 Cell culture

Cell culture was performed using standard procedures (atcc.org). For adherent cells, HeLa (CCL-2) and A549 (CCL-185) were obtained from American Type Culture Collection (ATCC) and maintained in DMEM and F-12K media containing 10% FBS at 37 °C and 5% CO₂, respectively. Cells were passaged at approximately 80% confluency. For coating purposes, living adherent cells (HeLa and A549) were removed from plate bottom using Trypsin-EDTA (0.25%) and then suspended in culture media. For suspension cells, HL-60 (CCL240) was obtained from American Type Culture Collection (ATCC) and maintained in IMDM media containing 10% FBS at 37°C and 5% CO₂. The media of HL-60 cell were changed every 3 days. For phagocytosis purposes, HL-60 cells were differentiated into
neutrophil-like cells by addition of 1.3% DMSO to the culture medium for 10 days.7

3.4.4 SupraCell construction

**Synthesis of SupraCells with ZIF-8 NPs coating.** Two million living mammalian cells were rinsed with 1X PBS, and then suspended in 500 μL of 400 μg/mL ZIF-8 NPs in 1X PBS solution. After 10 s vortex, 500 μL of 32 μg/mL tannic acid in 1X PBS solution were added with 30 s vigorous mixing. Then, the living mammalian cells with ZIF-8 NP coatings (SupraCell-ZIF-8) were then rinsed with 1X PBS twice, and stored in culture media.

**Synthesis of SupraCells with MIL-100(Fe) NPs coating.** Two million living cells were rinsed with 1X PBS and then suspended in 500 μL of 200 μg/mL MIL-100(Fe) NPs in 1X PBS solution. After 10s vortex and 1min incubation, 500 μL of 32μg/mL tannic acid in 1X PBS solution were added with 60 s vigorous mixing. Then, the living mammalian cells with MIL-100(Fe) NP coatings {SupraCell-MIL-100(Fe)} were then rinsed with 1X PBS twice, and stored in culture media.

**Synthesis of SupraCells with MET-3-Fe NPs coating.** Two million living mammalian cells were rinsed with 1X PBS, and then suspended in 500 μL of 400 μg/mL MET-3-Fe NPs in 1X PBS solution. After 10 s vortex, 500 μL of 32 μg/mL tannic acid in 1X PBS solution were added with 30 s vigorous mixing. Then, the living mammalian cells with MET-3-Fe NP coatings (SupraCell-MET-3-Fe) were then rinsed with 1X PBS twice, and stored in culture media.

**Synthesis of SupraCells with mSiO_2 NPs coating.** Protocol A): for amine-functionalized mSiO_2 NPs, before coating, the synthesized NPs were incubated in a tannic acid solution (0.4 mg/mL in 0.5X PBS) for 3 h and then washed with DI water twice. For living mammalian cells coating, Two million living cells were rinsed with 1X PBS and then suspended in 500 μL of 100 μg/mL MSNs in 1X PBS solution. After 10 s vortex and 1 min incubation, 500 μL of 12 μM benzene-1,4-diboronic acid in 1X PBS solution were added with 60 s vigorous mixing. Then, the living mammalian cells with mSiO_2 NP coatings (SupraCell- mSiO_2) were then rinsed
with 1X PBS twice, and stored in culture media.

Protocol B): for thiol-functionalized mSiO$_2$ NPs, the synthesized NPs were washed with DI water twice. For living mammalian cells coating, Two million living cells were rinsed with 1X PBS and then suspended in 500 μL of 100 μg/mL MSNs in 1X PBS solution. After 10 s vortex and 1 min incubation, 500 μL of 50 μg/mL 4-arm-PEG5K-SH and 50 μM H$_2$O$_2$ in 1X PBS solution were added with 60 s vigorous mixing. Then, the living mammalian cells with mSiO$_2$ NP coatings (SupraCell-mSiO$_2$) were then rinsed with 1X PBS twice, and stored in culture media.

**Synthesis of SupraCells with Fe$_3$O$_4$ NPs coating.** Before coating, the bare Fe$_3$O$_4$ NPs were incubated in a tannic acid solution (0.4 mg/mL in 0.5X PBS) for several hours and then washed with DI water twice. For living mammalian cells coating, two million living cells were rinsed with 1X PBS and then suspended in 500 μL of 100 μg/mL Fe$_3$O$_4$ NPs in 1X PBS solution. After 10s vortex and 1 min incubation, 500 μL of 12 μM benzene-1,4-diboronic acid in 1X PBS solution were added with 60 s vigorous mixing. Then, the living mammalian cells with Fe$_3$O$_4$ NP coatings (SupraCell-Fe$_3$O$_4$) were then rinsed with 1X PBS twice, and stored in culture media.

**3.4.5 Cell viability test**

Cell viability of the constructed SupraCells was assessed by CellTiter-Glo 2.0 Assay. Briefly, SupraCell samples were first diluted to the concentration of 50,000 cells/mL, and then 100 μL of the SupraCell samples were added into 96-well plate (White Opaque). After that, 100 μL of CellTiter-Glo 2.0 Reagent was dispensed into each well. The luminescence was recorded 10 minutes after addition of CellTiter-Glo 2.0 Reagent by a BioTek microplate reader. The Cell viability was calculated as a percentage of non-coated mammalian cells.
3.4.6 SupraCell shell controlled disassembly or dissociation

SupraCell-ZIF-8 or SupraCell-MIL-100(Fe) were rinsed with 1X PBS, and then suspended in 20 mM EDTA-PBS solution (pH 5.0) for different times (maximum time: 30 min) to allow the controlled disassembly of MOF NP exoskeleton. Then, the cells were rinsed with 1X PBS twice and stored in culture media.

3.4.7 Cell culture and proliferation test

Native HeLa cells and SupraCell-ZIF-8 were rinsed with 1x PBS and then incubated with culture media in eppendorf microtubes for 24 h. Under this condition, only few cells attach to the microtube wall and most of cells remain in suspension state. After 2 h or 24 h incubation, SupraCell-ZIF-8 was rinsed with 1x PBS, and then suspended in EDTA-PBS solution to remove the shell. After that, the cells were rinsed with 1x PBS twice and stored in culture media.

For imaging, native HeLa cells and SupraCell-ZIF-8 (stored 2 h or 24 h, followed by disassembly of the exoskeleton) maintained at the density of 100,000 cells/mL were seeded on glass substrates and then cultured at 37 °C and 5% CO₂. The cell samples were imaged using the Leica DMI3000 B inverted microscope.

For the cell proliferation test, native HeLa cells and SupraCell-ZIF-8 (stored 24 h followed by disassembly of the exoskeleton) were assessed by CellTiter-Glo 2.0 Assay. Briefly, 100 μL of cell samples at the density of 100,000 cells/mL were seeded in 96-well plates and then cultured at 37 °C and 5% CO₂. After that, 100 μL of CellTiter-Glo 2.0 Reagent was dispensed into each well. The luminescence was recorded 10 minutes after addition of CellTiter-Glo 2.0 Reagent by a BioTek microplate reader. The cell proliferation was calculated as a percentage of initial cell samples.
3.4.8 SupraCell mechanical characterization

To characterize the mechanical properties of the samples, a Triboindenter TI950 (Bruker-Hysitron) equipped with a standard 2D transducer and Berkovich tip was used. The tip was calibrated using a standard Fused Quartz sample for the required contact depth. To remove the surface roughness effects, the extracted curves with contact depths less than 50 nm have not been used for our data analysis. A rigid glass plate (E ~60 GPa) was used as the substrate for our indentation studies. The extracted stiffness and elastic modulus of the samples have been calculated according to the theory developed by Oliver-Pharr,⁸ and using the unloading section of the curves.

3.4.9 SupraCell Permeability test

The SupraCell permeability test was performed on SupraCell-MIL-100(Fe) toward a fluorescent glucose sugar of 2-NBDG and a nucleic acid (nuclear) staining dye of Hoechst 33342. Briefly, the native HeLa cells and supra-HeLa cells with MIL-100(Fe) coating were incubated with sugar of 2-NBDG (200 μM) and nuclear staining dye of Hoechst 33342 (3.2 μM) in cell culture media under cell culture condition for 1 h. After incubation, the cell samples were imaged using the Leica DMI3000 B inverted microscope.

3.4.10 SupraCell Cytoprotection test

Cytoprotection test to DOX. The cytoprotection test was performed on SupraCell-MIL-100(Fe). Briefly, the native HeLa cells and SupraCell-MIL-100(Fe) were seeded on a 96-well plate at the density of 200,000 cells/mL. Then different concentrations of DOX (0.01, 0.1, 1.0, 10, 20, and 50 μg/ML) were added to the cell culture media under cell culture conditions. After incubation for 2 h, the viability of the cells or SupraCells was measured by CellTiter-Glo 2.0 Assay.

Cytoprotection test to Ag NPs. The cytoprotection test was performed on SupraCell-MIL-100(Fe). Briefly, the native HeLa cells and SupraCell-MIL-100(Fe)
were seeded on a 96-well plate at a density of 200,000 cells/mL. Then, various solutions of different concentrations of Ag NPs (0, 4, 8, and 12 μg/ML) were added to the cell culture media under cell culture conditions. After incubation for 4 h, the viability of the cells or SupraCells was measured by CellTiter-Glo 2.0 Assay.

3.4.11 Phagocytosis assay

Phagocytosis studies of GFP-expressing salmonella typhimurium bacteria were performed in 10% FBS with DMSO free IMDM medium which was preheated to 37°C. Bacteria solutions were added to differentiated HL-60 cells and supra-HL-60-MIL-100(Fe) in a 100:1 bacteria/cells ratio, and then incubated for 1 h at 37 °C under rotation. Subsequently, both of the cells were rinsed with 1X PBS twice. Then the cells were incubated with 50 μg/mL gentamicin under cell culture condition for 30min to remove extracellular bacteria. After that, the cells were fixed in 3.7% formaldehyde in 1X PBS at room temperature for 10 min, rinsed with PBS, and then cellular filamentous actin network and nuclei were stained by rhodamine phalloidin and hoechst 33342, respectively. After staining, the cell samples were imaged using the Leica DMI3000 B inverted microscope.

3.4.12 SupraCell tolerance at harsh conditions

Tolerance to pH. Native HeLa cells and Supra-HeLa cell-MIL-100(Fe) were rinsed with saline solution (154 mM NaCl), and then suspended in saline solution at the density of 1,000,000 cells/mL. 20 μL of cell saline solution was added on the 96-well plate, and then 80 μL of different pH solutions with the same ion strength were dispensed into the well. The final pH value was adjusted to 4, 5, 6, 7.4, 8, 9, 10, or 11. The plate was then placed in an incubator at 37°C and 5% CO₂ for 1 h. After 1 h incubation, the viability of the cells was measured by the CellTiter-Glo 2.0 Assay.

Tolerance of ion strength. Native HeLa cells and Supra-HeLa cell-MIL-100(Fe) were rinsed with 1X PBS and then incubated in 0.25X PBS, 0. 5X PBS, 0.75X PBS, 1X PBS, 2X PBS, 3X PBS, 4X PBS, 5X PBS for 1 h, respectively. After
1 h incubation, the viability of the cells was measured by the CellTiter-Glo 2.0 Assay. For imaging purposes, the cell samples were fixed in 3.7% formaldehyde in the related PBS solution at room temperature for 10 min, rinsed with PBS, and then the cellular filamentous actin network and nuclei were stained with fluorescent probes of Alexa Fluor 488 phalloidin and Hoechst 33342, respectively. After staining, the cell samples were imaged using the Leica DMI3000 B inverted microscope and Leica TCS SP8 confocal laser scanning microscope. The cell counting was processed by Image Pro-Plus software.

**Tolerance toward ROS.** The ROS tolerance test was performed on Supra-HeLa cell-MIL-100(Fe) toward H$_2$O$_2$. Briefly, the native HeLa cells and Supra-HeLa cell-MIL-100(Fe) were rinsed with 1X PBS, and then suspended again in 1X PBS. Then, the cell samples were seeded on the 96-well plate at the density of 20,000 cells/well, and then incubated with different concentration of H$_2$O$_2$ (0, 2, 4, 6, and 8 mM) in 1X PBS solution at room temperature. After 1 h incubation, the viability of the cells was measured by the CellTiter-Glo 2.0 Assay.

**Tolerance toward UV exposure.** Native HeLa cells and Supra-HeLa cell-MIL-100(Fe) {or Supra-HeLa cell-MIL-100(Fe) with Congo red dye loading} were rinsed with 1X PBS, and then suspended again in 1X PBS. The cells were seeded on the UV transparent 96-well plate at the density of 200,000 cells/mL. The plate was placed in a home-made dark chamber equipped with compact UV Lamps (4 W lamps, Entela UL3101). The distance between the plate and the UV lamp was adjusted to be 5 cm.$^9$ After UV irradiation for 2 h (254 and 308 nm, respectively), the viability of the cells was measured by the CellTiter-Glo 2.0 Assay and LIVE/DEAD® Cell Imaging Kit.

### 3.4.13 SupraCell-Modular nanoparticle superassembly

Amine-functionalized mSiO$_2$ NPs with Fluorescein isothiocyanate, Rhodamine B isothiocyanate, and Alexa Fluor™ 633 NHS Ester (Succinimidyl Ester) labeling were used for modular nanoparticle superassembly. Before coating,
all the NPs were in a tannic acid solution (0.4 mg/mL in 0.5X PBS) for several hours and then washed with DI water twice. For SupraCell construction, Two million living cells were rinsed with 1X PBS and then suspended in 500 μL of 100 μg/mL mixed mSiO₂ (~1:1:1 ratio) in 1X PBS solution. After 10 s vortex and 1 min incubation, 500 μL of 12 μM benzene-1,4-diboronic acid in 1X PBS solution were added with 60 s vigorous mixing. Then, the living mammalian cells simultaneously coated with three kinds of mSiO₂ were then rinsed with 1X PBS twice, and stored in culture media.

3.4.14 SupraCell-Magnetic manipulation

Magnetic SupraCells were oriented and displaced in the application of an external magnetic field produced by a neodymium magnet. The bright field images were taken by Leica DMI3000 B inverted microscope to evaluate the magnetic manipulation.

3.4.15 SupraCell-In situ NO sensing

Supra-Raw 264.7-UiO-66-NH₂ was rinsed with 1X PBS and suspended cell culture media. The cells were seeded on the black 96-well plate at the density of 6,000,000 cells/mL. LPS solution was added to Supra-Raw 264.7@ UiO-66-NH₂ with a final concentration of 20 μg/mL. After that, Supra-Raw 264.7@UiO-66-NH₂ with or without LPS was incubated at 37 °C and 5% CO₂. The presence of NO was determined through fluorescence signals measured by a BioTek microplate reader with excitation at 370 nm and emission at 440 nm. All fluorescence measurements were performed at room temperature.

3.4.16 SupraCell Conductivity measurement

Supra-Hela cell-MET-3 (Fe) was deposited on conductive indium tin oxide coated glass slides. For electrical measurements, Keithley 6487 picoammeter/voltage source was retro-fitted inside of FEI Quanta 3D FEG Dual Beam (SEM/FIB) (FEI, Hillsboro, OR, USA) to control and measure the current and
voltage between the probe and substrate (Figure S3.36). Probe tips are polycrystalline tungsten wire electrochemically etched to an end radius of curvature of less than 250 nm.
3.5 References


CHAPTER 4

Armored red blood cells: multifunction integration through modular super-assembly of hybrid nanoparticle-based building blocks

This chapter was adapted from Jimin Guo‡, Wei Zhu‡*, Rita E. Serda, Jacob Ongudi Agola, Achraf Noureeddine, Evelyn Ploetz, Stefan Wuttke* and C. Jeffrey Brinker* Armored red blood cells: multifunction integration through modular super-assembly of hybrid nanoparticle-based building blocks. (‡Authors contributed equally to this work)
4.1 Introduction

Red blood cells (RBCs; also called erythrocytes), are the most abundant cellular constituent of blood and a natural drug delivery system in vertebrates.\[1\] Erythrocytes are special cells in various aspects: they are biconcave shaped, isolated cells without organelles, which exclusively serve as biological carriers. They only exist to distribute and transport various compounds contained within their volume and extended membrane surface. RBCs are responsible for oxygen delivery (O\(_2\)) throughout the body. They show prolonged circulation through the vascular system for up to 3 months within mammals and have access to not only components within the blood,\[1\] but also the endothelium and reticuloendothelial system (RES) under physiological conditions. RBCs play a pivotal medical role in various fields including transfusion medicine, or the regulation of the adaptive immune system (e.g. by carrying anti-inflammatory agents or inhibitors of phagocytes). Due to their physiological impact and properties, including biocompatibility, abundance and longevity in circulation, RBCs have been explored over the past decades as carriers of various compounds and nanoparticles and served as an inspirational source of novel functional assemblies and advanced architectures for biomedical applications\[2-6\]. Examples range from coupling drugs onto the surface of erythrocytes to improve their delivery and therapeutic effects\[7-9\], to the attachment of nanoparticles (NPs) onto RBC membrane to alter the circulation behavior of NPs\[10-13\], the encapsulation of RBCs with nanometric films to modulate immune response\[14-18\], and the embedding of magnetic NPs in the interior of RBCs to enable magnetic alignment and guidance\[19-20\].
To date, different strategies have been developed for RBC engineering: 1) Genetic engineering - causing RBCs to express therapeutic proteins for the treatment of different diseases\textsuperscript{[21]}; 2) Surface grafting - that modifies the RBC membrane by coupling of drugs/targeting agents\textsuperscript{[22]}; 3) Hypotonic loading - that employs formation of transient pores in plasma membranes in hypotonic solutions to allow the subsequent loading of drugs or NPs in the RBC inner volume\textsuperscript{[2-3]}; 4) Surface hitchhiking - where functional NPs are noncovalently attached to the membrane\textsuperscript{[10-13]}; 5) Cell-in-shell – that describes approaches based on the encapsulation of RBCs by a nanometric artificial shell \textit{e.g.} made of polyelectrolytes, polydopamine, or iron-phenolic networks\textsuperscript{[14-18,23]}.

Nevertheless, RBCs remain highly sensitive and instable, biological structures, that strongly depend on the environment such as the tonicity of chemicals and reaction conditions as well as handling, which strongly hampers current RBC engineering approaches. In order to truly turn RBCs into multifunctional supernanocarrier with externally tunable functions and properties, several limitations needs to be overcome: Current protocols are limited by (\textit{i}) multiple processing steps and/or long preparation time, (\textit{ii}) continuous need of optimization of most material synthesis conditions (such as pH, temperature, precursor concentration) to avoid RBC lysis, and (\textit{iii}) the lack of versatile capability to endow RBCs with multiple functionalities for biomedical applications.

To overcome these limitations, we have developed a general engineering strategy to generate stabilized, multifunctional RBC-based supercarriers that are externally tunable based on a superassembly approach where nanometric metal-
organic frameworks (MOFs) acting as functional building blocks. MOFs are periodic and atomically well-defined porous crystalline materials that are typically self-assembled by metal nodes and organic ligands, offering structural diversity, high surface area, tunable porosity, and due to their hybrid nature, the ability to independently functionalize the external and internal surfaces\(^{[24-26]}\). This enables researchers to design MOFs for a spectrum of applications including gas storage and separation\(^{[27-28]}\), water harvesting\(^{[29-30]}\), sensing\(^{[31]}\), energy\(^{[32]}\), drug delivery\(^{[33-35]}\), and acting as nanobuilding blocks for the construction of complex hierarchical nanoarchitectures\(^{[36-37]}\).
Figure 4.1 | Modular functionalization of RBCs with MOF nanobuilding blocks. a, Schematic representation of Armored RBC formation via immediate, multidentate ligand (tannic acid) assisted super-assembly of MOF nanobuilding blocks on RBC membrane surface. Schematic illustration of the properties of Armored RBCs: in vivo circulation (b), oxygen delivery (c), particle hitchhiking and redistribution (d), and enhanced resistance against external stressors, blood NO sensing, and potential drug delivery (e).

Inspired by the well-defined and modular chemistry of MOFs, here we introduce the concept of ‘Armored Red Blood Cells’ which are RBCs encapsulated and surface engineered with functional, modular, MOF nanobuilding block-based exoskeletons (Figure 4.1). Exoskeletons are constructed within seconds through fast MOF NP super-assembly based on strong-multivalent metal-phenolic coordination[37,38] and RBC/MOF complexation via multiple hydrogen-bonding interactions at the cellular interface (Figure 4.1a). The developed simple universal coating approach is highly biocompatible. It does not introduce RBC hemolysis nor affect the normal physiology of RBCs in terms of e.g., oxygen-carrying capability or circulation behavior, as confirmed by the presence a reversible oxygenated and deoxygenated state and long circulation times as determined in chicken embryo and mice models, respectively. Depending on the type of MOF NPs or NP combinations, the physico-chemical properties (e.g. optical, magnetic, and sensing properties) of Armored RBCs are highly tunable. The potential chemical diversity of Armored RBCs is enormous, and here various functional MOF NPs including ZIF-8, MIL-100 (Fe), UiO-66-NH₂, magnetic iron oxide (Fe₃O₄) NPs@ZIF-8, and
hybrid mesoporous silica NP@MOF (MSNs, dye-labeled MSNs, sensing probe-loaded MSNs@ZIF-8), have been prepared for diverse Armored RBC prototype construction. The newly created Armored RBCs not only show enhanced tolerance against external stressors such as antibody-mediated agglutination, detergent/toxic NPs caused lysis, osmotic stress, and freezing, but also possess RBC abiotic properties including controlled disassembly, multi-fluorescence, magnetism, and blood nitric oxide (NO) sensing, which are utterly foreign to the native RBCs. Taken together, the versatile RBC coating strategy holds great promise to promote the design of new MOF/ RBC-inspired functional microarchitectures for a wide range of bioapplications.

4.2 Results and Discussion

4.2.1 Modular super-assembly of Armored RBCs

The presented approach of Armored RBCs has a striking advantage for the design of multifunctional, hierarchical nano-assemblies employed for drug delivery and molecular imaging. It can revert to the full range of metal organic framework nanoparticles, as functional, robust and modular building blocks. When constructing a multifunctional, protective shell around single RBCs, these MOF-based NP form a fast exoskeleton based on particle-particle super-assembly and interlocking at the proximal RBC membrane surface. The exoskeleton assembly occurs in two steps: at first, MOF NPs attach and concentrate onto the native RBC surface. Since RBC membranes are rich in carbohydrates and proteins, they have a highly negatively charged surface\(^{[39]}\). Due to the frangibility and sensitivity of
RBCs, a strong interaction between NPs and RBC membranes always causes RBC rupture and hemolysis. MOF NP surfaces comprise well-defined, long periodic arrangements of metal nodes and organic ligands, which allow precise tuning of the coordination and interactions with organic moieties on the RBC membrane surface. Based on zeta potential (ζ) measurements, MOF NPs used within this study (UiO-66-NH₂, MIL-100(Fe), and ZIF-8) have a negative charge ranging from -3.0 to -29.1 mV (Figure S4.1), which should avoid strong electrostatic interactions between the MOF NPs and the negatively charged RBC surfaces (zeta = ~30 mV) thereby suppressing hemolysis; however, the electrostatic repulsion may limit MOF accumulation and attachment. To balance both contributions, we carefully chose an isotonic buffer (phosphate-buffered saline (PBS) at pH 5.0), in which the zeta potential of MOF NPs is greatly decreased and no hemolysis of RBCs occur. Under these conditions, hydrogen bonding interactions between the organic ligands from MOF NPs and carbohydrates and proteins from RBCs drive MOF accumulation and attachment. The second step of exoskeleton assembly of Armored RBCs involves interlocking of MOF NPs, that are already attached to the RBCs surface and additional exogenous MOFs via an interparticle ligand. We employed tannic acid as the interparticle ligand and added it sequentially to the MOF NPs/RBCs mixed solution after short time incubation (~30 s). Tannic acid is frequently used as an organic building block in MOFs, due to its well-known biodegradability and strong-multivalent coordination binding to various metal ions. Coupled with the additional
strong metal-phenolic interactions, the colloidal MOF NP-based exoskeleton in armored RBCs can be created rapidly, in seconds.

As a demonstration of the Armored RBC concept, individual purified RBCs (Figure 4.2a) encapsulated within UiO-66-NH₂ MOF NP-based exoskeletons (termed Armored RBC-UiO-66-NH₂) were constructed via the sequential addition of a colloidal UiO-66-NH₂ NP solution and tannic acid to RBC suspensions (See materials and methods in SI for detailed information). Colloidal UiO-66-NH₂ MOF NPs with diameter of ~ 440 nm were synthesized according to the reported solvothermal methods[41]. Transmission and scanning electron microscopy (TEM, SEM) (Figure 4.2b) and wide-angle X-ray diffraction (XRD) (Figure 4.2f) confirmed their crystalline structures with well-defined octahedral shape. The formation of UiO-66-NH₂-based exoskeletons around RBCs is clearly visible not only by SEM (Figure 4.2c) but can be even observed by the naked eye (Figure S4.2) as a color change from bright red to light red. The roughened surface (Figure 4.2c), which is due to a dense packing of NPs at the RBC surface, is further visible in bright field images (Figure S4.3). Fourier-transform infrared spectroscopy performed on Armored RBC-UiO-66-NH₂ (Figure S4.4) confirmed the presence of UiO-66-NH₂ MOF NPs, as evidenced by the characteristic peaks at 1570 and 1256 cm⁻¹ assigned to the –CO₂ asymmetrical stretching and vibration of C–N, respectively, in the aminocarboxylate groups of UiO-66-NH₂. Analyzing nearly fifty Armored RBCs on bright field optical (Figure S4.3) and SEM images (Figure 4.2c) supported the fact that all erythrocytes were encapsulated within homogeneous conformal exoskeletons. This could be further confirmed by confocal scanning
laser microscopy of fluorescein isothiocyanate-labeled Uio-66-NH₂ NP-based exoskeletons. A uniform and homogeneous Uio-66-NH₂-NP layer that encapsulates the erythroid cells can be observed (Figure 4.2d). Moreover, energy-dispersive X-ray (EDX) spectroscopy for mapping of zirconium, oxygen, and nitrogen atoms (Figure 4.2e) along with wide-angle XRD (Figure 4.2f) further confirmed, that the structural and chemical integrity of Uio-66-NH₂-NPs are preserved within the exoskeleton. Importantly, the fast coating process did not cause the hemolysis of RBCs even after 7 days storage (Figure 4.2g), indicating that the formed exoskeleton has no toxic effects on RBCs.

Figure 4.2 | Armored RBC structure characterization. a, Bright field (left) and SEM (right) image of RBCs. Scale bars, 25 μm (left), 2 μm (right). b, TEM and SEM image of Uio-66-NH₂ MOF NPs. Scale bars, 50 nm (left), 500 nm (right). c, SEM image of the armored RBCs-Uio-66-NH₂. Scale bar, 5 μm. d, Confocal fluorescent image of armored RBCs-Uio-66-NH₂ labeled
with fluorescein isothiocyanate. Scale bars, 5 μm. 

e, SEM mapping (Zr, O, and N) of armored RBCs-UiO-66-NH₂. Scale bars, 5 μm. 
f, Wide XRD patterns of the synthesized armored RBCs-UiO-66-NH₂ and the simulated UiO-66-NH₂ crystals. 
g, Time-dependent hemolysis of armored RBCs@UiO-66-NH₂ in 1x PBS solution and RBCs in water as control.

To demonstrate the generality of the Armored RBC approach, i.e. the protective and functional encapsulation of native RBCs by diverse types of MOF NPs via spontaneous super-assembly, we tested MIL-100(Fe), magnetic Fe₃O₄ NPs@ZIF-8, and hybrid MSNs@ZIF-8 in different shapes, sizes and functionalities as building blocks for the design of various Armored RBCs. The full characterization and description of each of these armored RBC types appears below. The results clearly demonstrate that the Armored RBC approach is a powerful and universal strategy to create multifunctional, cellular super-assemblies, as it is a fast, easy, and biocompatible functionalization process of cellular membranes.

4.2.2 Armored RBCs show enhanced resistance against external stressors

The first impressive property of Armored RBCs is their enhanced cytoprotection against external stressors. To benchmark the protective effect, we exposed armored RBC-MIL-100(Fe) to various harsh environmental conditions including antibody-mediated agglutination, osmotic pressure, detergents, toxic NPs, and freezing conditions (Figure 4.3a). The bright field image in Figure S4.5 confirmed the successful assembly of a homogeneous MIL-100(Fe)-NP-based exoskeleton. At first, antibody-mediated agglutination assays were performed.
(Figure 4.3b) to assess the blood group antigen immunogenicity of Armored RBCs. This is a crucial factor in RBC alloimmunization\cite{14}, since a cross-type agglutination reaction caused by blood type mismatch during blood transfusion may lead to potentially fatal massive immune hemolysis and even patient death. As shown in Figure 4.3b (top row), native RBCs of type B rapidly and severely agglutinated in presence of small amounts of anti-A serum. Identical results were obtained with type A and type RhD-RBCs in their corresponding anti-type anti-sera; on the contrary, no antibody-mediated aggregation was observed in Armored RBCs even with the extension of exposure time (Figure 4.3b - bottom row). Armored RBCs possess a highly effective immune-protective exoskeleton that shields the immune-response provoking epitopes on RBC surfaces against agglutination. Our novel protection strategy may allow the survival of foreign RBCs during blood transfusion and thus can act as potential universal RBCs that overcome the blood type mismatch problem. Secondly, the tolerance of Armored RBCs to osmotic pressure was tested, and the percentage of cells undergoing hemolysis measured. At first glance, the osmotic fragility curves of both native RBCs and Armored RBCs showed similar rupture profiles (Figure 4.3c). Cellular fractures of native and Armored RBCs were initiated at a concentration of NaCl of 0.60% (w/v), but native RBCs bursted completely at a concentration of 0.30%, while the burst concentration of Armored RBCs was shifted to 0.20%. The enhanced tolerance to osmotic pressure can be attributed to two sources / contributions.
Figure 4.3 | Enhanced resistance of Armored RBC to harsh environmental conditions.

a, Schematic illustration of the protection of RBCs against external stressors based on MOF NP encapsulation. 

b, The optical images of human type A, B, and Rh RBCs in their corresponding anti-typing sera. Scale bar, 50 μm. 

c-e, Hemolytic behavior of native RBCs and Armored RBCs-MIL-100(Fe) as a function of (c) NaCl concentration (i.e. osmotic pressure
stimulus), (d) Triton X-100 concentration (i.e. detergent stimulus), and (e) Stöber particle concentration (i.e. NP stimulus). Scale bar is 300 nm. 

The recovery of native RBCs cryopreserved in HES polymer (175 or 215 mg mL\(^{-1}\)) PBS dispersions, and Armored RBCs cryopreserved in PBS solution and protected by MIL-100(Fe) NP-based exoskeletons with increasing coating cycles.

On one hand, the enhanced membrane reinforcement provided by the MOF exoskeleton offers a physical restriction that delays the swelling process. On the other hand, the potential adsorption of ions in MOF pores may delay the ion transfer from external medium to RBC intracellular fluids\(^{[42]}\). Both effects could lead to a reduced and delayed RBC lysis under hypotonic condition. Next, the hemolytic protection of Armored RBCs exposed to the nonionic detergent Triton X-100 and so-called ‘Stöber’ amorphous silica NPs was tested, since both species are known to easily cause RBC lysis. As shown in Figure 4.3d-e, a slight change in Triton X-100 concentration or particle concentration greater than 500 μg mL\(^{-1}\) already led to massive lysis of RBCs, whereas Armored RBCs exhibited negligible hemolysis under both conditions. We attribute this cytotoxic agent resistance to a physical barrier effect provided by the armored shell. Finally, we determined the resistance of Armored RBCs to freezing conditions in comparison to native RBCs. Ice recrystallization is the major challenge during cryopreservation of RBCs\(^{[43]}\). The formation of ice crystals not only incites serious mechanical damages to the delicate RBCs but also creates increased osmotic pressure across the cell membrane, leading to rupture of RBCs. In order to provide extensive ice recrystallization and maximize cell stress during the cryopreservation test, both native RBC and Armored RBC samples were rapidly frozen in liquid nitrogen (\(\sim\)196
ºC) for 2 h and then slowly thawed at 4 ºC over several hours. As shown in Figure 4.3f, the native RBC recovery in PBS solution is very low (< 5 %). However, protected by the armored shell, the cell recovery increased to ~25 %. With further increase of coating cycles to get a thicker shell, the cell recovery of RBCs can be increased up to 40 % without the addition of any toxic solvents. This cell recovery is superior to that obtained via commonly used hydroxyethyl starch polymers (HES) at concentration of 17.5 wt% and 21.5 wt%, highlighting the excellent protection conferred by the MOF NP-based exoskeletons.

4.2.3 Assessment of Armored RBCs with respect to their oxygen carrier capability, ex ovo and in vivo circulation and biodistribution

An important feature of RBCs is their oxygen carrier capability and long-circulation times in blood. To facilitate the in vivo bioapplication of our newly designed Armored RBCs, Armored RBCs must exhibit the very same behaviors as native RBCs. At first, we employed luminol-based chemiluminescence to reveal the presence of hemoglobin in Armored RBCs (Figure 4.4a). When hemoglobin and luminol-perborate mixture come in contact, the iron in the hemoglobin accelerates the reaction of luminol with the peroxide generated from perborate, which results in a bluish glow (Inset of Figure 4.4b). After addition of luminol-perborate mixture in solution, both native RBCs and Armored RBCs-MSN@ZIF-8 become chemiluminescent after 10 min. This clearly shows that the armored shell does not inhibit the iron catalytic property of hemoglobin inside RBCs. The inherent porosity of the armored shell provides full accessibility of small molecules such as luminol and peroxide to the RBC and permits access to and crossing of the RBC
membrane. To investigate the oxygen carrier capability, UV-Vis spectroscopy was used to reveal the reversible shift of maximum absorption peaks of RBCs in oxygenated and deoxygenated states (Figure 4.4c and Figure S4.6). The characteristic absorption peak of native RBCs and Armored RBCs in an oxygenated state appeared at 415 nm (Figure 4.4c and Figure S4.6). After bubbling nitrogen for 2 h and adding the reducing agent sodium dithionite (Na$_2$S$_2$O$_4$), the absorption peak red-shifts to 430 nm, confirming the switch to the deoxygenated state for both native RBCs and Armored RBCs.

Figure 4.4 | Assessment of Armored RBC oxygen carrier capability and ex ovo circulation. a, Schematic illustration of the oxygen binding via hemoglobin by RBCs. b, Time-
dependent oxygenation curves of native RBCs and Armored RBC-MSN@ZIF-8. Insert images show the generation of bluish glow of native and Armored RBCs after the addition of luminol-perborate mixture. c, UV-Vis spectra of the oxygenated and deoxygenated states of Armored RBC-MSN@ZIF-8 (top), and the related reversible transfer between two states (bottom). The circulation of Armored RBC-MSN@ZIF-8 (d) and native RBCs (e) in the vessel of a chick embryo in the chorioallantoic membrane (CAM model (left), and the related (confocal) fluorescence images (right). Scale bars are 50 μm (left), 5 μm (right). f, The flow of Armored RBC-MSN@ZIF-8 within the CAM capillary bed. Scale bar is 20 μm.

The deoxygenated state can carry oxygen again after exposure to atmospheric oxygen. The process of binding and releasing oxygen was repeatable (Figure 4.4c), demonstrating that the oxygen carrier capability of Armored RBCs was preserved. By comparing the oxygenation rate of both deoxygenated samples, as shown in Figure 4.4b, the time-dependent oxygenation curves of native and Armored RBCs showed the similar behaviors, while a ~60 s delay was found for Armored RBCs. We explain this observation by the presence of the porous MOF shell that increases the tortuosity of the oxygen diffusion pathway, and thus causes oxygen molecules to spend more time trapped within MOF pores.

RBCs are well known to easily traverse the microvasculature with dimensions that are smaller than their size and display long circulation times in vivo. To investigate the circulation behavior of Armored RBCs, we carried out real-time fluorescence wide-field imaging on a chick embryo ex ovo (chorioallantoic CAM model; Figure S4.7), that provides easy and direct optical access for intravital imaging of the flow of RBCs in blood vessels. Alexa Fluor 647-labeled MSN@ZIF-8 hybrid NPs with diameter of ~80 nm were used as building blocks for
Armored RBC construction (Figure S4.8). A coherent, conformal NP layer encapsulating RBCs, clearly observed in Figure 4.4d, confirmed the successful formation of Armored RBCs. The blood vessels are labeled with fluorescein labeled lens culinaris agglutinin (LCA) for direct visualization. As shown in Figure 4.4d,f, compared to the flow of the native RBCs in the blood vessels and capillaries (Figure 4.4e and Figure S4.9), the flow of the Armored RBCs was not affected, even after circulating for 30 min, supporting normal circulation properties, despite the Armored RBCs shape exhibiting a certain degree of deformation compared to native RBCs.

To further characterize circulation properties of the Armored RBCs in vivo, we used albino C57BL/6 mice to examine their pharmacokinetic and biodistribution behaviors. Mice were injected with control DyLight 800-labeled MSN@ZIF-8 hybrid NPs and the corresponding MSN@ZIF-8 Armored RBCs by retro-orbital injection, respectively,[44] at a dose of 150 μg NPs/mouse. Syngeneic RBCs were used to create Armored RBCs, negating blood cell type complications. To study the circulation half-life, at various time points following the injection (Figure 4.5a), blood was collected from the eye socket of the mice to evaluate the concentrations of circulating control NPs or Armored RBCs. At 12 h and 24 h post injection, the Armored RBCs exhibited 19 % and 15 % overall retention in mice blood, respectively, as compared to the 6 % and 2 % exhibited by control NPs (Figure 4.5d). The semilog plot of retention-circulation time (Figure S4.10) illustrates a bi-exponential decrease in particle concentration over time, indicating that both NP and Armored RBC circulation followed a two-compartment pharmacokinetic
After fitting to the two-compartment pharmacokinetic model, numerical analysis (Figure 4.5d) indicated that the elimination half-life of NPs and Armored RBCs was 16.0 ± 3.7 h and 66.3 ± 17.4 h, respectively. Armored RBCs displayed a remarkably enhanced retention in blood circulation in comparison with control NPs. Our finding is well correlated with the reports that the anchoring of particles onto the RBC surface could prolong the intravascular particle circulation, where the flexibility, circulation, and vascular mobility of RBCs could help the adhered nanoparticles to escape rapid reticuloendothelial system (RES) clearance[10-13].

Furthermore, to analyze the related biodistribution, at 12 h and 24 h post injection, mice were euthanized and their liver, spleen, kidneys, heart, lungs, and blood were harvested for fluorescence analysis (Figure 4.5b). The majority of fluorescence signal was found in the two primary filtering organs, the liver and spleen after 12 h post injection, supporting removal by the RES. However, the fluorescence intensity from the Armored RBCs in the spleen decreased 24 h post injection and simultaneously increased in the lungs, suggesting detachment of MOFs from the RBC surface and their return into circulation (Figure 4.5a). Notably, 24 h after injection, the Armored RBCs exhibited a 10-fold higher blood persistence and 3.5-fold higher accumulation in lungs compared to the control NPs (Figure 4.5e-f). It was postulated that NPs from Armored RBCs accumulate in the lungs following release caused by squeezing through tiny capillaries in the lung vasculature[10-13]. The high cardiac blood output can cause shear forces that could facilitate the transfer of the NPs from RBC surface to pulmonary capillary endothelial cells.
Figure 4.5 | Armored RBCs circulation and biodistribution assessment.  

a, Whole mice fluorescence images acquired using the IVIS Spectrum at 0.5, 1, 2, 6, 12, and 24 h after intravenous administration of DyLight 800-labeled MSN@ZIF-8 hybrid NPs (top) and the related Armored RBCs (bottom), respectively. Fluorescence images of different organs (liver, spleen, kidney, heart and lung from top to bottom) at 12 and 24 h after intravenous administration of NPs (b) and Armored RBCs (c), respectively. d, Circulation time of both NPs and Armored RBCs (n = 3; mean ± SD). Insert table is the related elimination half-life. Fluorescence intensity per gram of tissue (e) and relative fluorescence signal per organ (f) at 12 and 24 h after intravenous administration of NPs and Armored RBCs (n = 3; mean ± SD).

In summary, our newly created Armored RBCs exhibit enhanced in vivo circulation/residence times compared to many other classes of nanoparticles\textsuperscript{10-13} and could serve as sources of MOFs (or conceivably other nanoparticles) that could be released and targeted to various organs over time. This could extend the
in vivo applications of MOFs and enable targeting and delivery to difficult-to-reach sites in the body.

4.2.4 Multifunctional Armored RBCs construction

Given the chemical diversities of MOF nanobuilding blocks, the armored RBC concept can be generally extended based on a plethora of MOF types and combinations. It is basically unlimited in generating diverse functionalities and hence serves as a promising technology to satisfy the growing need of multifunctional nanoparticles in biomedical applications, which we demonstrate in the following section. Depending on the MOF building block, armored MOF shells can (i) not only be created but also be biocompatible disassembled if necessary. Depending on the underlying MOF NPs, armored RBCs offer (ii) unique physiochemical properties such as optical, magnetic and sensing properties. But most importantly: (iii) the armored RBC concept profits enormously from multiplexing: MOF NP associated properties can be linearly combined within the Armored RBC shell during a mixed super-assembly synthesis.

Armored RBCs hold great promise for drug delivery applications. In order to function as a Trojan Horse, the encapsulation shell of Armored RBCs plays a pivotal role. Not only their synthesis, but also their triggered disassembly enables Armored RBCs to serve as a nanoparticle reservoir and source fulfilling the Trojan Horse concept. Our newly designed armored RBCs, based on UiO-66-NH$_2$ MOF NPs exhibit this behavior. Their shell can be disassembled in ethylenediaminetetraacetic acid (EDTA) solution in a programmed fashion due to the responsive nature of metal-phenolic complexation. The armored MOF shell can be
progressively disassembled over the time course of 15 min (as shown by fluorescence microscopy for UiO-66-NH₂-covered RBCs labeled with fluorescein isothiocyanate (Figure 4.6a)).

Figure 4.6 | Multifunctional Armored RBCs as tools in nanomedicine. a, Controlled disassembly of the shell leads to a reversible recovery of the RBC normal state for Armored RBC-UiO-66-NH₂ labeled via fluorescein isothiocyanate. The etching time in presence of EDTA amounted to 0, 5 and 15 min. Scale bars, 5 μm. b-c, Armored RBCs serving as a sensor. b, Schematic illustration of the sensing of NO within blood vessels based on the use of the fluorescent probe of DAR-1. c, Calibration curve: fluorescent intensity change versus NO concentration. The red star points out the NO concentration in fresh blood based on the sensing function of DAR-1 loaded Armored RBCs. Insert fluorescent image is DAR-1-loaded Armored RBCs after incubation in NO solution (100 μM) for 5 min. d-e, Armored RBCs as imaging contrast agent. d, Schematic illustration of the manipulation of magnetic Armored
RBCs via an external magnetic field (left). Photographs of a dispersion of magnetic Armored RBCs before (left) and after (right) placing a magnet on its side. e, Bright-field microscopy images of magnetically-actuated Armored RBC-Fe₃O₄@ZIF-8 (left) or immobile RBCs (right). Scale bars, 10 μm. f, Fluorescence spectra of multifluorescent Armored RBCs-MSN@ZIF-8 with emission at 428, 515, 648 nm. g, Multimodal armored RBCs. Confocal images of multi-fluorescent Armored RBCs based on three different fluorescent MSN@ZIF-8 nanobuilding blocks. Scale bar, 5 μm.

Note that the EDTA etching solution (20 mM, pH 7.4) has a negligible impact on RBCs. Upon EDTA-induced detachment of MOF NPs for 15 min, the armored MOF shells were almost completely removed and no defects were observed on the RBC surface suggesting that the RBCs can reversibly return to their original morphology. This on-demand protection shell formation and degradation capability provides RBCs a novel capability reminiscent of the germination of natural spores.

To demonstrate the sensor capabilities of Armored RBCs, we employed the modular properties of MOF NPs to design Armored RBCs that detect nitric oxide (NO) in blood. NO is a key signaling molecule acting as a potent vasodilator that relaxes the arteries (Figure 4.6b)[45]. Hybrid MSN@ZIF-8 NPs (Figure S4.8) were used as exoskeleton building blocks wherein 4,5-Diamino-rhodamine B (DAR-1), serving as a fluorescent probe, was pre-loaded within the MSN mesopores. NO-triggered fluorescence was observed for the DAR-1 probe, after its ring-closure and transition from the weakly fluorescent diamino structure to the strongly fluorescent triazole state[46]. The sensitivity of designed DAR-1-loaded Armored RBCs was determined by exposing the encapsulated cells to freshly prepared NO
solutions with different NO concentrations. The fluorescence intensity increases monotonically with increasing NO concentration (Figure 4.6c), which we determined as integrated fluorescence over the sample cuvette via fluorescence wide field imaging of DAR-1-loaded Armored RBCs (see for example, Figure 4.6c (Inset) at 100 µM NO and 5 min incubation time). By incubating our sensing Armored RBCs in fresh blood for 5 min, the NO concentration in fresh blood could be determined to be 8.6 nM, which is in line with literature values[47]. This study demonstrates the potential of our designed Armored RBCs as sensors not only for real-time monitoring and detection of NO in blood, but also further measurements like cellular pH or ROS and redox potentials depending on chosen fluorophores[48].

Armored RBCs inherit the collective properties of their MOF or other NP building blocks imparting non-native desirable properties. To demonstrate this idea, we created magnetic Armored RBCs that can be externally controlled. Based on metal-phenolic linker chemistry, magnetic Fe₃O₄ (~8.0 nm) embedded in ZIF-8 MOFs (Figure S4.11) were super-assembled onto the RBC surface to form a magnetic armored shell. In contrast to native RBCs, the magnetic armored RBCs can now be manipulated via an external magnetic field (Figure 4.6d-e). This property is not only of interest for 3-dimensional cell patterning and micro-motorized cellular constructs[19-20], but also magnetic targeting and controlled delivery of therapeutic agents[49]. Moreover, the controllable magnetic switching makes multifunctional Armored RBCs clearly a potential candidate for hyperthermia induction and a contrast reagent for clinical tumor and leukemia detection via medical MRI imaging[50].
Finally, modular super-assembly of Armored RBCs provides far-reaching possibilities for extensions via co-assembly of different functional MOF- (or NP-) based nano-objects into multimodal nano-structures. These could integrate various, simultaneous functionalities, such as contrast for different imaging modalities, thermal therapies and drug delivery. To introduce Armored RBCs as multimodal super-architecture, we created multi-fluorescent RBCs by incubating native RBCs simultaneously with almost equal concentrations of three different fluorescently labelled MSN@ ZIF-8 NPs in a one-pot process for less than 2 minutes (Figure 4.6f-g). The fluorescence spectra of resulting Armored RBCs featured three distinct emission peaks at 428, 515 and 648 nm (Figure 4.6f). Confocal fluorescence microscopy images in 3D demonstrated the formation of a continuous exoskeleton and a homogeneous distribution of NPs that preserved the stoichiometry of the synthesis solution (Figure 4.6g). This example suggests that the co-assembled Armored MOF shell could introduce a nearly infinite number of functionalities to Armored RBCs and might provide efficient coupling effects between the functional MOF NPs.

4.3 Conclusion

We have developed a general, simple, and modular approach to create a novel class of hybrid biomaterials termed Armored RBCs with diverse possible functionalities. Using metal-phenolic chemistry we encapsulated native RBCs with MOF NP-based exoskeletons in seconds without RBC lysis. The modularity and simplicity of this method arises from fast MOF NPs super-assembly at the RBC membrane surface and enables the transformation of different MOF building
blocks and RBC vehicles into diverse functional hierarchitectures. The presented Armored RBCs preserve the original properties of native RBCs, show enhanced resistances against external stressors, and exhibit extraordinary new properties that are foreign to native RBCs based on the highly modular nature of MOF nanobuilding blocks integrated into the RBC exoskeletons. The presented approach profits from the wide range of variable MOF NPs and opens the door to design of multimodal nano-superstructures for multimodal imaging, image-guided therapies and theranostics. The strategy of Armored RBCs, however, is not restricted to red blood cells alone, but can be further extended to any other cell types like leucocytes playing a pivotal role in immunity and inflammatory processes. We believe our findings will open new avenues for artificially designed cell-inspired functional materials for wide ranging biomedical applications.
4.4 Materials and Methods

4.4.1 General information

**Reagents.** All chemicals and reagents were used as received. Zinc nitrate hexahydrate, 2-methylimidazole, zirconium(IV) chloride, 2-aminoterephthalic acid, dimethylformamide (DMF), iron(III) chloride hexahydrate, trimesic acid, tetraethyl orthosilicate (TEOS), (3-aminopropyl)triethoxysilane (APTES), ammonium hydroxide, ammonium nitrate, hexadecyltrimethylammonium bromide (CTAB), cyclohexane, tannic acid, ethylenediaminetetraacetic acid, fluorescein isothiocyanate (FITC), iron(III) acetylacetonate (Fe(acac)₃), benzyl alcohol, methanol, Ham's F-12K (Kaighn's) medium, Iscove's modified Dulbecco's media (IMDM), formaldehyde solution (36.5-38% in H₂O), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Alexa Fluor™ 647 NHS ester (succinimidyl ester) and DyLight 800 NHS ester were purchased from Thermo Fisher Scientific. Heat-inactivated fetal bovine serum (FBS), 10X phosphate-buffered saline (PBS), 1X trypsin-EDTA solution, and penicillin-streptomycin (PS) were purchased from Gibco (Logan, UT). Dulbecco’s modification of Eagle’s medium (DMEM) was obtained from Corning Cellgro (Manassas, VA). Absolute ethanol was obtained from Pharmco-Aaper (Brookfield, CT; 200 proof). Milli-Q water with a resistivity of 18.2 MΩ cm was obtained from an inline Millipore RiOs/Origin water purification system.

**Characterization methods.** Scanning electron microscopy (SEM) analyses and energy-dispersive X-ray spectroscopy (EDS) elemental mappings were performed on a Hitachi SU-8010 field-emission scanning electron
microscope at 15.0 kV. Transmission electron microscopy (TEM) and high-resolution TEM (HRTEM) imaging were carried out using a Hitachi model H-7650 transmission electron microscope at 200 kV. Wide-angle powder X-ray diffraction (PXRD) patterns were acquired on a Rigaku D/MAX-RB (12 kW) diffractometer with monochromatized Cu Kα radiation (λ = 0.15418 nm), operating at 40 KV and 120 mA. The UV-Vis absorption spectra were recorded using a Perkin-Elmer UV/vis Lambda 35 spectrometer. Fluorescence emission measurements were carried out using a fluorescence spectrometer (Perkin-Elmer LS55). Three-color images were acquired using a Zeiss LSM510 META (Carl Zeiss MicroImaging, Inc.; Thornwood, NY, USA) operated in channel mode of the LSM510 software.

4.4.2 Nanoparticles synthesis

UiO66-NH₂ NPs synthesis. UiO-66-NH₂ NPs were synthesized following previously reported methods¹ with no modification. Briefly, 25.78 mg ZrCl₄ (0.11 mmol) and 14.49 mg 2-aminoterephthalic acid (0.08 mmol) were dissolved in 10 mL of DMF solution. Then 1.441 g acetic acid (0.024 M) was added into the above solution. The mixed solution was placed in an oven (120 °C) for 24 h. After cooling down the reaction mixture to room temperature, obtained NPs were subsequently washed with DMF and methanol via centrifugation redispersion cycles. The synthesized UiO-66-NH₂ NPs were stored in EtOH before use.

MIL-100(Fe) NPs synthesis. MIL-100(Fe) NPs was synthesized following previously reported methods with no modification.² Briefly, 2.43 g iron(III) chloride hexahydrate (9.0 mmol) and 0.84 g trimesic acid (4.0 mmol) in 30 ml H₂O were mixed in a Teflon tube, sealed, and placed in the microwave reactor (Microwave,
Synthos, *Anton Paar*. The temperature of the mixed solution was increased from room temperature to 130 °C under solvothermal conditions (P = 2.5 bar) within 30 seconds, and then kept at 130 °C for 4 minutes and 30 seconds, and finally cooled down again to room temperature. The synthesized NPs were centrifuged down and then washed twice with EtOH. The dispersed NPs were allowed to sediment overnight. The remaining supernatant of the sedimented suspension was filtrated (filter discs grade: 391, *Sartorius Stedim Biotech*) three times to finally yield the required MIL-100(Fe) NPs. The synthesized MIL-100(Fe) NPs were stored in EtOH before use.

**Mesoporous silica NPs (MSN) synthesis.** MSN NPs was synthesized following previously reported methods in our group with no modification. ³ Briefly, 0.29 g of CTAB (0.79 mmol) was dissolved in 150 mL of 0.51 M ammonium hydroxide solution in a 250 mL beaker, sealed with parafilm (Neenah, WI), and placed in a mineral oil bath at 50 °C. After continuously stirring for 1 h, 3 mL of 0.88 M TEOS solution in EtOH and 1.5 μL APTES were combined and added immediately to the mixed solution. After another 1 h of continuous stirring, the particle solution was stored at 50 °C for another 18 h under static conditions. Next, the solution was passed through a 1.0 µm Acrodisc 25 mm syringe filter (PALL Life Sciences, Ann Arbor, MI) followed by a hydrothermal treatment at 70 °C for 24 h. To remove the CTAB, the synthesized MSN NPs were transferred to 75 mM ammonium nitrate solution in ethanol, and placed in an oil bath at 60 °C for 1 h with reflux and stirring. The MSN NPs were then washed in 95% ethanol and transferred to 12 mM HCl ethanolic solution and heated at 60 °C for 2 h with reflux.
and stirring. Finally, MSN NPs were washed in 95% ethanol, then 99.5% ethanol, and stored in 99.5% ethanol before use.

**Fe$_3$O$_4$ NPs synthesis.** Bare Fe$_3$O$_4$ NPs was synthesized following the reported methods with no modification. Briefly, 0.687 g of Fe(acac)$_3$ (1.94 mmol) was dissolved in 9 mL of benzyl alcohol. The mixed solution was heated to 170 °C with reflux and stirring at 1500 rpm for 24 h. After the reaction was cooled down to room temperature, 35 mL EtOH was added into the mixed, and then centrifuged at 20000 rpm for 10 min. The supernatant was discarded, and the resulted precipitate was washed with EtOH twice to yield to the required Fe$_3$O$_4$ NPs. The synthesized Fe$_3$O$_4$ NPs were stored in EtOH before use.

**MSN@ZIF-8 NPs synthesis.** 2.5 mg MSNs were suspended in 2.5 mL water. Next, 250 μL of Zn(NO$_3$)$_2$ (0.134 M) and 1 mL of 2-MIM (0.219 M) were subsequently added into the solution. The mixed solution was stirred for 0.5 h, and then centrifuged at 20000 rpm for 10 min. The supernatant was discarded, and the resulted precipitate was washed with EtOH twice to yield to the required NPs. The synthesized MSN@ZIF-8 NPs were stored in EtOH before use.

**Fe$_3$O$_4$@ZIF-8 NP synthesis.** 2.5 mg Fe$_3$O$_4$ were suspended in 2.5 mL water and then 250 μL of Zn(NO$_3$)$_2$ (0.134 M) and 1 mL of 2-MIM (0.219 M) was subsequently added into the solution. The mixed solution was stirred for 0.5 h, and then centrifuged at 20000 rpm for 10 min. The supernatant was discarded, and the resulted precipitate was washed with EtOH twice to yield to the required NPs. The synthesized Fe$_3$O$_4$@ZIF-8 NPs were stored in EtOH before use.
4.4.3 RBC Purification

All the animal procedures complied with the guidelines of the University of New Mexico Institutional Animal Care and Use Committee and were conducted following institutional approval (Protocol 11-100652-T-HSC and 17-200658-HSC). Human RBCs were acquired from healthy donors with their informed consent. All blood samples were collected and stored in BD Vacutainer® blood collection tubes (Becton Dickinson, NJ, USA) containing 1.5 mg of EDTA per mL of blood for anticoagulation purposes. The purification of whole blood was carried out using Ficoll® density gradient centrifugation procedure.\(^5\)

4.4.4 Armored RBC construction

**Synthesis of Armored RBC-UiO-66-NH\(_2\).** 5 million RBCs were suspended in 500 μL of 1X PBS (pH 5) solution containing 400 μg/mL UiO-66-NH\(_2\) NPs. After 10 s vortexing and 30 s of incubation, 500 μL of 40 μg/mL tannic acid in 1 X PBS (pH 5) solution was added with 30 s vigorous mixing. The formed Armored RBC-UiO-66-NH\(_2\) was then rinsed with 1X PBS (pH 7.4), and stored in 1X PBS (pH 7.4).

**Synthesis of Armored RBC with MIL-100(Fe) NPs coating.** 5 million RBCs were suspended in 500 μL 1X PBS (pH 5) solution containing 200 μg/mL MIL-100 NPs. After 10 s vortexing and 20 s of incubation, 500 μL of 32 μg/mL tannic acid in 1X PBS (pH 5) solution were added with 30 s vigorous mixing. The formed Armored RBC-MIL-100(Fe) was then rinsed with 1X PBS (pH 7.4), and stored in 1X PBS (pH 7.4). This process represents a typical procedure for single
MIL-100(Fe) NP shell formation and could be repeated one or two times to achieve multi-layered coating.

**Synthesis of Armored RBC-MSN@ZIF-8.** 5 million RBCs were suspended in 500 μL 1X PBS (pH 5) solution containing 400 μg/mL MSN@ZIF-8 NPs. After 10 s vortexing and 20 s incubation, 500 μL of 32 μg/mL tannic acid in 1X PBS (pH 7.4) solution were added with 30 s vigorous mixing. The formed Armored RBC-MSN@ZIF-8 was then rinsed with 1X PBS (pH 7.4), and stored in 1X PBS (pH 7.4).

**Synthesis of Armored RBC-Fe₃O₄@ZIF-8.** 5 million RBCs were suspended in 500 μL 1X PBS (pH 5) solution containing 250 μg/mL Fe₃O₄@ZIF-8 NPs. After 10 s vortexing and 20 s incubation, 500 μL of 40 μg/mL tannic acid in 1X PBS (pH 7.4) solution were added with 20 s vigorous mixing. The formed Armored RBC-Fe₃O₄@ZIF-8 was then rinsed with 1X PBS (pH 7.4), and stored in 1X PBS (pH 7.4).

**4.4.5 Hemolysis assay**

Native and Armored RBCs were rinsed with 1X PBS (pH 7.4) solution and then suspended in 1X PBS (pH 7.4) solution at room temperature for 7 days. After centrifugation (300 g, 5 min), the absorbance of hemoglobin in the supernatant was measured by a BioTek microplate reader (Winooski, VT) at 540 nm to calculate the hemolysis percentage. Double distilled (D.I.) water and 1X PBS (pH 7.4) solution containing native RBCs were used as positive control (100% hemolysis) and negative control (0% hemolysis), respectively. The hemolysis percentage of each sample was determined using the reported equation.³ Percent
hemolysis (%) = 100 \times \frac{\text{Sample Abs}_{540\text{nm}} - \text{Negative control Abs}_{540\text{nm}}}{\text{Positive control Abs}_{540\text{nm}} - \text{Negative control Abs}_{540\text{nm}}}

4.4.6 Antibody-mediated agglutination

The Antigenic protective capability of Armored RBC was assessed by investigating the attenuation of antibody-mediated agglutination of RBCs. Briefly, 1 million native RBC or Armored RBC-MIL-100(Fe) samples were suspended in 450 μL 1X PBS (pH 7.4) solution, and then 50 μL of anti-type sera that included A, B, and Rh were added. After 15 min, the bright field images were taken by Leica DMI3000 B inverted microscope to evaluate the agglutination.

4.4.7 Tolerance against ion strength

Native and Armored RBC-MIL-100(Fe) were rinsed with 1X PBS (pH 7.4) solution and then suspended in different concentration of NaCl solution (0 to 0.8%, w/v). After centrifugation (300 g, 5 min), the absorbance of hemoglobin in the supernatant was measured by a BioTek microplate reader (Winooski, VT) at 540nm to calculate the hemolysis percentage.

4.4.8 Tolerance against detergent

Native RBCs and Armored RBC-MIL-100(Fe) were rinsed with 1X PBS (pH 7.4) solution and then incubated in 1X PBS (pH 7.4) with different concentrations of Triton X-100. After centrifugation (300 g, 5 min), the absorbance of hemoglobin in the supernatant was measured by a BioTek microplate reader (Winooski, VT) at 540 nm to calculate the hemolysis percentage.
4.4.9 Tolerance against toxic nanoparticles

Native RBCs and Armored RBC-MIL-100(Fe) were rinsed with 1X PBS (pH 7.4) solution and then incubated in 1X PBS (pH 7.4) with different concentrations of Stöber sphere silica NPs at room temperature for 3 h in continuous rotating state. After centrifugation (300 g, 5 min), the absorbance of hemoglobin in the supernatant was measured by a BioTek microplate reader (Winooski, VT) at 540 nm to calculate the hemolysis percentage.

4.4.10 Cryopreservation and cell recovery

The cryopreservation was tested by referring to the reported paper with slight modifications. Hydroxyethyl starch (HES) were dispersed in 1X PBS (pH 7.4) solution with the concentration of 175.0 and 215.0 mg/mL. 50 million/mL native RBCs and Armored RBC-MIL-100(Fe) were rinsed with 1X PBS (pH 7.4) solution and then suspended in 1X PBS (pH 7.4) solution or HES solution. Each sample was frozen by immersion in liquid nitrogen (−196 °C) for 2 h prior to thawing. Thawing of samples was undertaken by transferring samples to 4 °C in the fridge for a minimum of 2.5 h. Slow thawing process promoted extensively ice recrystallization while ensuring samples were fully thawed. Next, the samples were centrifuged (300 g, 5 min) and the absorbance of hemoglobin in the supernatant was measured by a BioTek microplate reader (Winooski, VT) at 540 nm to calculate the cell recovery. Double distilled (D.I.) water and 1X PBS (pH 7.4) solution containing native RBCs were used as the positive and negative controls,
respectively. The cell recovery percentage of each sample was determined using the same equation given in section S5.

4.4.11 Chemiluminescence

Luminol-Based chemiluminescence was used to evaluate oxygen carrying capacity of RBCs. Briefly, 70 mg sodium perborate, 500 mg sodium carbonate, and 200 mg luminol were added to 5 mL water and dissolved by sonication. The luminol solution was left undisturbed for 5 min in a dark room. For imaging purposes, 1 mL of luminol solution was added to 4 mL samples (20 million native RBC or Armored RBCs) in 1X PBS (pH 7.4) solution. The optical image was taken by a Sony ILCE-5100 Camera (ISO-100 and exposure time of 1/15 s). The chemiluminescence optical image was taken in a dark room by a Sony ILCE-5100 Camera (ISO-6400 and exposure time of 30 s). For luminescence assay, 100 μL of samples in 1X PBS (pH 7.4) solution were added into white 96-well plates at a density of 5 million cells/mL. After that, 20 μL of luminol solution was added to each well and the contents were mixed for 2 min on shaker in the dark. Luminescence was measured using a BioTek microplate reader and expressed as relative quantity.

4.4.12 Capability of reversibly binding oxygen

Capability of reversibly binding oxygen was detected by analyzing changes of UV–Vis absorption spectra (300–700 nm) in oxygenated and deoxygenated solutions. For complete deoxygenation, nitrogen gas was in flown into the sample solution to deplete most of the oxygen. After 2 h, sodium dithionite (Na₂S₂O₄) was
added, and UV–Vis absorption spectrum was scanned by a BioTek microplate reader. For oxygenation, sample solutions were exposed to atmospheric oxygen for more than 2 h, and UV–Vis absorption spectrum was recorded. This process represents the typical procedure for reversibly binding oxygen capability. Technical replica amount to 3.

4.4.13 Test of vascular flow in Ex ovo chick embryos

The vascular flow characteristics of Armored RBCs were tested using Ex ovo Chick embryo model as described previously\(^3\) and was conducted following institutional approval (Protocol 11-100652-T-HSC). Briefly, eggs were acquired from East Mountain Hatchery (Edgewood, NM) and placed in a GQF 1500 Digital Professional incubator (Savannah, GA) for 3 days. Embryos were then removed from shells by cracking into 100 ml polystyrene weigh boats. Ex ovo chick embryos were covered and incubated at 37 °C, 100% humidity. 20 million cells/mL of native RBCs and Alexa Fluor 647-labeled-Armored RBC-MSN@ZIF-8 were incubated in 1X PBS (pH 7.4) solution with 10 mg/mL bovine serum albumin (BSA) for 20 min and then rinsed and stored in 1X PBS (pH 7.4) solution. 100 μL of samples in 1X PBS (pH 7.4) solution were injected into secondary or tertiary veins via pulled glass capillary needles. Embryo chorioallantoic membrane (CAM) vasculature was imaged using a customized avian embryo chamber and a Zeiss Axio Examiner upright microscope with heating stage.
4.4.14 *In vivo studies on pharmacokinetics and biodistribution*

All animal procedures complied with the guidelines of the University of New Mexico Institutional Animal Care and Use Committee and were conducted following institutional approval (Protocol 17-200658-HSC). The experiments were performed on female Albino C57BL/6 mice (6 weeks). To evaluate the circulation half-life of NPs and Armored RBCs, DyLight 800-labeled MSN@ZIF-8 hybrid NPs and the related Armored RBCs were used. Briefly, both samples were incubated in 1X PBS (pH 7.4) solution with 10 mg/mL bovine serum albumin (BSA) for 30 min and then rinsed and stored in 1X PBS (pH 7.4) solution. 150 μL of NPs (1mg/mL) and the related Armored RBCs (1 mg/mL NPs on Armored RBCs) were injected into the eye of the mice. The blood was collected at 0.5, 1, 2, 6, 12, and 24 h following the injection. Each time point group contained three mice. The collected blood samples were diluted with the same amount of 1X PBS before the fluorescence measurement. Particle retention in circulation at these time points was determined by measuring the fluorescence on a BioTek microplate reader (Winooski, VT). Pharmacokinetics parameters were calculated to fit a two-compartment model. To study the biodistribution of the NPs and Armored RBCs in various tissues, 150 μL of NPs (1 mg/mL) and the related Armored RBCs (1 mg/mL NPs on Armored RBCs) were retro-orbital injected to mice. At 12 and 24 h time points following the particle injection, three mice were randomly selected and euthanized. Their liver, spleen, kidneys, heart, lung, and blood were collected. The collected organs were examined with an IVIS fluorescence imaging system.
(Xenogen, Alameda, CA), and the fluorescence intensity of the NPs and Armored RBCs in different organs was further semi-quantified by the IVIS imaging software.

### 4.4.15 Armored RBC shell controlled destruction

The Armored RBCs were rinsed with 1X PBS and then suspended in 20 mM EDTA in 1X PBS (pH 7.4) solution for different times (maximum time: 15 min) to allow the controlled destruction of MOF NPs. Then the RBCs were then rinsed with 1X PBS (pH 7.4), and stored in 1X PBS (pH 7.4).

### 4.4.16 NO sensing

Preparation of NO solution followed a reported protocol in NO sensor studies.\(^8\) 10 mM NaOH and 1X PBS (pH 7.4) solutions were pre-bubbled with nitrogen for 2 h to deplete the dissolved oxygen. NO precursor Diethylamine NONOate sodium salt was added to a 10 mM NaOH solution to make the 500 μM stock solution. The stock solution was diluted with 1X PBS (pH 7.4) solutions to generate various concentrations of NO solutions. The NO-containing PBS solutions were set for at least 15 min to allow the NO concentrations to saturate before NO sensor studying. For *in vitro* NO study, 2.5 million DAR-1-loaded Armored RBCs were suspended in 1 mL of NO-containing PBS solution. For fresh blood NO study, the collected flash blood samples were first diluted with the same amount of 1X PBS, and then incubated with 1 mL solution containing 2.5 million sensing Armored RBCs. After 5 min incubation, the fluorescence emission spectrum was obtained.
4.4.17 Armored RBC magnetic manipulation

The magnetic Armored RBC-Fe$_3$O$_4$@ZIF-8 were oriented in the direction of an external magnetic field produced by a neodymium magnet. The bright field images were taken by Leica DMI3000 B inverted microscope to evaluate the magnetic guidance.

4.4.18 Armored RBC modular nanoparticles super-assembly

Three different fluorescently labelled MSN@ZIF-8 NPs were used for modular nanoparticles superassembly. For Armored RBC construction, 5 million RBCs were suspended in 500 μL of 400 μg/mL mixed MSN@ZIF-8 nanoparticles (~1:1:1 ratio) in 1X PBS (pH 5) solution. After 10 s vortex and 20 s incubation, 500 μL of 32 μg/mL tannic acid in 1X PBS (pH 7.4) solution were added with 30 s vigorous mixing. The formed multi-fluorescent Armored RBCs were then rinsed with 1X PBS (pH 7.4), and stored in 1X PBS (pH 7.4).
4.5 References


CHAPTER 5

Biomimetic Rebuilding of Multifunctional Red Blood Cell: Design Using Functional Modules

This chapter was adapted from

5.1 Introduction

The integral functioning of cells remains a poorly understood subject due to the inherent complexity and fragility of biological systems.\textsuperscript{[1]} To overcome these challenges, artificial cells are designed to simplify and mimic functions of the natural cell as well as provide a platform to incorporate abiotic features not innate to the native cells.\textsuperscript{[2]} This kind of design can be illustrated by the red blood cell (RBC), which given its low biologic complexity (e.g. lack of cell nucleus and most intracellular organelles\textsuperscript{[3]}), is a suitable target for biomimicry and rebuilding of functional artificial cells. While biologically simple, RBCs possess unique potential focal points for biomimicry such as special biconcave discoidal shape that provides a favorable surface area-to-volume ratio and allows RBCs to undergo remarkable flexible deformations, ability to carry oxygen through the formation of oxyhemoglobin complex, and presence of multiple molecular biomarkers on the membrane that ensure recognition as self by the immune surveillance system, leading to long circulation times.\textsuperscript{[4-6]}

There have been several attempts to generate artificial RBC-like materials by mimicking the key structural and functional features of RBCs. Mitragotria, et al.\textsuperscript{[4]} developed synthetic biomaterial particles that closely mimic the shape, flexibility, and the ability to carry oxygen similar to the natural RBCs. Similarly, DeSimonea, et al.\textsuperscript{[5]} explored the effect of mimicking RBC shape and flexibility on the vascular circulation time and biodistribution characteristics. While these studies presented encouraging findings, their overriding shortcoming was a predominant focus on one or two aspects of the RBC’s unique characteristics, rather than a broad
spectrum of RBC properties that can lead to a multifunctional RBC-mimicking material. In addition, neither of these studies considered the unique properties of the RBC membrane and how they impact long-term circulation times. In this study, a modular design approach was used to rebuild a multifunctional artificial RBC particle that fully mimics most of the broad properties of native RBCs and performs additional abiotic functions not inherent to the native RBCs. This modular design strategy is based on the use of three building blocks of silica cell bio-replication, layer-by-layer self-assembly process and encapsulation with the RBC derived membrane ghosts (Scheme 5.1). It is envisioned that this rebuilt RBC (RRBC) platform will provide a better understanding of the relationship between structure and functions of RBCs, and further advance our understanding of complex living systems.
Scheme 5.1. Schematic illustration of the design and construction of rebuilt RBC via silicification of RBC, calcination, polyion polymer coating, crosslinking, silica etching, and RBC membrane ghost fusion (A) and the properties of rebuilt RBC (B): circulation and oxygen delivery (a), cargo delivery (b), and detoxification and toxin sensing(c).

5.2 Results and Discussion

5.2.1 Construction of Rebuilt RBCs

In order to construct the RRBC particle, four major steps (Scheme 5.1) were used; 1) preparation of the silica RBC replica, 2) polymer coating and silica removal to create the RBC-polymer replica, 3) functional cargo loading, and 4) fusing of the purified RBC membrane ghost onto the surface of RBC-polymer replica to yield a micron-size RRBC particle. As detailed in the methods section, the silica RBC replica was prepared through the silica cell bio-replication process, which translates the native RBCs into inorganic silica materials under mild acidic conditions.\[^{7,8}\] To construct the RBC-shaped polymer core, the silica RBC replica was used as a template for the layer-by-layer self-assembly deposition of biocompatible complementary polyelectrolytes (positively charged chitosan and negatively charged alginate polymers) onto the silica RBC replica surface through electrostatic interactions (Figure S5.1). Both chitosan and alginate are natural polysaccharides and were used in this process due to their biomedical application relevant features of low toxicity-allergenicity, high biocompatibility and,
The aminopolysaccharide chitosan layers were cross-linked using glutaraldehyde to provide stability to the RRBC particles.

**Figure 5.1.** A) Scanning Electron Microscopy (SEM) images of silica RBC replica with polymer. Scale bars, 2μm. B) Fluorescence image of RBC-membrane-derived ghost. Scale bars, 10μm. C) Differential interference contrast (DIC) images of rebuilt RBC. Scale bars, 2μm. D) DIC images of rebuilt RBC. Scale bars, 10μm. E) Zeta potential of the synthetic steps to create rebuilt RBC. The zeta potential measurements for RBC* and RRBC* was acquired in 154 mM NaCl solution, the rest were measured in 5 mM NaCl solution. F-H) Confocal microscopy images of rebuilt RBC. Scale bars, 20μm. The inset images are of higher magnification of rebuilt RBC. Scale bars, 3μm.
The polymer-coated silica RBC replica (Figure 5.1A) was then suspended in buffered hydrofluoric (HF) acid solution to etch the silica (Figure S5.1 and S5.2) and yield RBC-polymer replica, which due to its high surface charge, provides a platform for loading functional cargos through electrostatic interactions. The RBC-membrane ghost was obtained by treating fresh RBCs with a hypotonic solution to remove intracellular or cytosolic contents with minimal perturbations to the cell membrane (Figure 5.1B). The membrane ghost was then subsequently mixed with RBC-polymer replica and sonicated in ice water bath (~ 0 °C) for 1 min, so that the shear force imposed by the sonication process could rupture the RBC membrane ghosts and facilitate the fusion of the membrane ghosts onto the surface of the RBC-polymer replica, yielding the final RRBC particle.

Different strategies were used to characterize the RRBC particle during the construction process. Zeta potential (ζ) measurements were obtained to assess fluctuations of the surface charge during the synthetic steps from the native RBCs (ζ = -2.9 mV) to the final RRBC particle. Upon silicification and subsequent calcination to yield silica RBC replicas, the zeta potential values changed from -2.9 to -8.0 mV (Figure 5.1E) consistent with the translation of the native RBC surface to the silanol groups (Si-OH) of the silica surface. Upon adding the polyion complex to the silica RBC replicas to generate RBC-polymer replica, a further reduction in zeta potential from -8.0 to -27.0 mV was observed (Figure 5.1E), consistent with the contributions of the negatively charged carboxylic acid groups (COOH) present in the alginate. However, after fusing RBC membrane ghosts onto the polymer core surface, there was an increase in zeta potential from -27 to -
17mV (Figure 5.1E), attributed to the charge screening effects of the RBC membrane ghosts and the negatively charged sialyl moieties on the RBC extracellular membrane side.\cite{11,12} Furthermore, when transferred to the biologically relevant solution, 154 mM NaCl, utilized to assess the native RBC zeta potential, the RRBC zeta potential (-3.4 mV) closely matched that of the native RBC (-2.9 mV) (Figure 5.1E).

In order to verify the RRBC particle’s structure, fluorescein isothiocyanate (FITC) modified chitosan was used to prepare the RBC-shaped polymer core and then fluorescent 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (18:1 Liss Rhod PE) lipid was mixed with RBC-membrane ghosts and then fused onto the polyion template. The resulting dual-labeled RRBC particle was then visualized using confocal fluorescence microscopy. In Figure 5.1, microscopy images show that different building blocks of RRBC particles overlap in the same location, revealing an intact core-membrane structure of the RRBC particles with the encapsulating RBC membrane ghost. In addition, the images also unequivocally reveal that RRBC particles could maintain the characteristic biconcave discoid shape typical of the native RBCs, which provides excess surface area and ultimately gives rise to the majority of the mechanical and transport properties of the RBCs by enabling necessary extreme shape deformation.\cite{7,8}

5.2.2 Deformability of Rebuilt RBCs

Having overcome the challenges inherent in designing the biconcave discoid shape of the RBCs, the softness and deformability behavior of the RRBC
particle was then examined. Flexibility-deformability of the native RBCs is one of the key properties that enables RBCs to easily traverse the microvasculature with dimensions smaller than their size and display long circulation times in the body.\textsuperscript{[4-6,13,14]} A microfluidic blood capillary model\textsuperscript{[15,16]} designed using physiologically relevant dimensions and pressure drops that commonly occur in blood capillaries,\textsuperscript{[15,16]} was used to investigate the flow-based deformation of the RRBC particles (\textbf{Figure 5.2A and 5.2B}). RRBC particles were injected from a arterial inlet, while the control buffer (1x PBS) was injected from a venous inlet (\textbf{Figure 5.2C}) of the capillary model. Since the flow rate of each inlet could be tuned independently, the pressure drops across the capillaries could easily be controlled. To tune the stiffness of the RRBC particles and enable their passage through the small capillary, different concentrations of the glutaraldehyde crosslinker were used to link the polymer layers of the RBC-shaped polymer core, based on the positive correlation between particle stiffness and changes in the amount of crosslinker used \textsuperscript{[4-6,15,16]}. It was observed that, the highly cross-linked RRBC particles could not pass through the model capillaries at lower pressure differentials (\textbf{Figure 5.2F}), due to higher stiffness and lower deformation capacity while on the contrary, RRBC particles with lower cross-linking levels could undergo the requisite deformation and pass through the model capillaries (\textbf{Figure 5.2G}), confirming that softness and flexibility are important properties needed for the RRBC particles to traverse smaller dimension microvasculature. Furthermore, the less cross-linked RRBC particles were able to regain their discoidal shape upon exiting the capillary flow.
system (Figure 5.2D), confirming the reversibility of the RRBC particle shape deformation characteristic of the native RBCs.

Figure 5.2. Schematics of the blood capillaries (A) and microfluidic blood capillary model (B). C) Illustration of the available flow paths for rebuilt RBC and control particles in the microfluidic blood capillary model, overlaid on the bright field image of microfluidic blood capillary model. Scale bars, 100μm. D) Bright field image of rebuilt RBC at the outlets of the microfluidic device after passing through the small dimension (5μm) capillaries. Scale bars, 5μm. Note that the rebuilt RBC were not visibly damaged by the deformation. E) Trajectory analysis of rebuilt RBC
(cross-linker concentration of ~4%, 8%, and 12%) and controls (silica RBC replicas and RBCs). This analysis was done by dividing the number of particles passing through the capillaries by the number of particles directly through the arterial side. Mean ± standard deviation, n = 3. F, G) Time-lapse fluorescence microscopy images of rebuilt RBC (grey) stopping (cross-linker concentration ~12%, F) or passing through (cross-linker concentration ~4%, G) the microfluidic device capillaries. Scale bars, 5μm. The pressure drop across the capillaries is ∼6 mbar in F and G. The “drawn out” shape of the rebuilt RBC observed in some of the time-lapse frames is due to the acquisition rate of the imaging camera. The biconcave shape is again observed when the rebuilt RBC are no longer in motion (as seen in C).

The trajectories of the particles in the microfluidic blood capillary device were further analyzed to quantify the deformability behavior (Figure 5.2E). This was done by calculating the ratio of the number of particles passing through the capillaries relative to the number of particles remaining on the sample side,[15,16] based on the principle that, the particles that could easily pass through the capillaries would have a higher ratio, while the particles that could not pass through would have a zero ratio.[15,16] A decrease in glutaraldehyde crosslinker concentration resulted in decreased stiffness of the RRBC particles, and a corresponding increase in softness and deformation capacity (Figure 5.2E). As expected, even at higher pressure drops, the control silica RBC replica could not pass through the capillaries, possibly due to the stiffness of silica network. Further, RRBC particles with 4% crosslinker concentration and the native RBCs had similar deformability behavior in the microfluidic blood capillary model, confirming that RRBC particles at this crosslinker concentration may display similar in vitro circulation properties as the native RBCs.
5.2.3 Surface properties of Rebuilt RBCs

Having confirmed the deformability characteristic of the RRBC particles in the capillary model, their membrane properties were then assessed. The membrane of the native RBC is highly oriented with asymmetric distribution of phospholipids, glycans, and proteins between the intracellular and extracellular sides.\textsuperscript{17-19} The glycans and proteins on the extracellular side, play important roles in phagocytic cell recognition and internalization.\textsuperscript{12,17-19} Similarly, right-side-out-membrane orientation of the RRBC particle is critical for this construct to maintain native RBC membrane-like properties.\textsuperscript{12} In order to verify the membrane orientation of the RRBC particle, antibody-mediated agglutination assay was performed to detect the classical AB(O) and Rh antigens (\textbf{Figure S5.3}). While AB(O) are glycosphingolipid antigens protruding from the RBC membrane, Rh is a lipoprotein antigen embedded in the RBC membrane.\textsuperscript{17-19} Commercially sourced anti-A, anti-B, and anti-RhD sera were used to examine the antigens\textsuperscript{17-19} on the membranes of both the native RBC and RRBC particle. When treated with their respective anti-type sera, both the native RBC and the RRBC particle rapidly agglutinated (\textbf{Figure S5.3}), whereas no agglutination occurred when they were treated with non-matching anti-type sera (\textbf{Figure S5.3 and S5.4}). This was attributed to the preservation of the RBC membrane biomarkers together with the right-side-out membrane orientation. In addition, the presence and surface membrane availability of the ICAM-4, an erythroid-specific membrane glycoprotein important in the RBC’s interactions with the macrophage cells,\textsuperscript{20} was also confirmed by the fluorescent anti-ICAM-4 antibody (\textbf{Figure S5.5}), which further
speaks to the RRBC particle’s membrane protein preservation and the right-side-out membrane orientation. In addition to the self-antigens, the presence of immunosuppressive proteins (e.g. CD47) was also assessed.\cite{11-12} CD47 is a transmembrane protein whose presence on the surface of the RBC membrane inhibits macrophage phagocytosis of RBCs and ensures long circulation times.\cite{11-12} Using FITC conjugated anti-CD47 antibody, the presence of CD47 protein on the membrane of the RRBC particles was confirmed (\textbf{Figure S5.5}), which together with the self-antigens and ICAM-4, indicates that the status of the immunosuppressive proteins was not perturbed/destroyed by the construction of the RRBC particles. Thus, the presence of these immunosuppressive proteins on the RRBC particle membrane should lead to long \textit{in vivo} circulation times within the vascular network\cite{11-12} and possibly ensure better delivery of the intended cargos.

\textbf{5.2.4 Biocompatibility and circulation properties of Rebuilt RBCs}

To demonstrate that the RRBC particles could display vascular flow characteristics in living systems, \textit{Ex ovo} chick chorioallantoic membrane (CAM)\cite{21-22} and mouse models were used coupled with appropriate imaging techniques. Before testing the vascular flow behavior of the RRBC particles, their biocompatibility characteristics were first assessed using hemolysis\cite{21-22}. In the hemolysis assays, it was noticed that only silica RBC replica exhibited significant hemolytic activity compared to the etched silica or polymer coated particles at biologically relevant concentrations (\textbf{Figure S5.6}). However, when a very high concentration of the test materials (~4×10^7 particles/mL) was used, only the
complete RRBC particle did not cause hemolysis while the rest of the test materials exhibited varying levels of hemolytic effects (Figure S5.6), confirming that bare silica and to a lesser extent alginate surfaces are toxic to red blood cells in vitro, while fusion with RBC ghosts abolishes this toxicity. Following confirmation of biocompatibility with native RBCs, cytotoxicity to other cells likely to be encountered by RRBC was assessed. Human umbilical vein endothelial cells (HUVEC) and mouse macrophage Raw264.7 cells were utilized for these assays. Cytotoxicity tests revealed no loss of cell viability in both HUVEC and Raw264.7 cells after exposure to RRBC particles, congruent with the hemolysis test results (Figure S5.6) and corroborating the overall high biocompatibility of the RRBC particles. These findings are significant because upon intravenous injection, biomaterials establish direct contact with the blood vessels which makes it necessary to understand their biocompatibility with the endothelial cells that cover the blood vessel lumen. [23]

Having established that the RRBC particles display excellent biocompatibility properties, their vascular flow characteristics within living systems were then assessed. Using the ex-ovo chick embryo (CAM) and direct intravital imaging[21-22], it was observed that, while the RRBC particles were able to easily circulate within the deep blood vessels of the Ex ovo chick embryo, both the RBC-polymer replica without native RBC membrane coating and silica*-RRBC (silica RBC replica@polymer-RBC ghost, without etching) were rapidly arrested in the CAM capillary bed (Figure 5.3G and 5.3H), due to the combined roles of the native red blood cell membrane properties and the stiffness caused by the silica network.
Importantly, it should be noted that, the circulation of the RRBC particles was only possible when they were closely matched in physical properties to the native RBCs through a process of successive silica etching followed by fusion of the native RBC membrane ghosts (Figure 5.3) to generate a complex with sufficiently low modulus and native RBC membrane like properties capable of withstanding inherent challenges of long-term circulation times.
Further characterization of the vascular flow properties of RRBC particles was carried out by examining the pharmacokinetic behavior and biodistribution profiles of the RRBC particles in a mouse model. In order to avoid the potential immune responses caused by the blood type non-conformity, the RRBC particles used for this characterization were prepared using syngeneic mice RBCs. FITC-labeled RRBC particles (3×10^7 particles/mL, 100 μL) were administered by retro-orbital injection. Blood was collected at 24 and 48 h post-injection, the RBC-membrane-coated particles exhibited 35% and 21% overall retention in the mouse blood respectively (Figure 5.4A). The semilog plot of the retention-circulation time illustrates a bi-exponential decrease in RRBC particle concentration over time (Figure 5.4A), indicating that the RRBC particle circulation follows a two-compartment pharmacokinetic model,[11-12] where the particles first distribute from the plasma into various tissues, followed by a late (elimination) phase and the ultimate clearance from the blood. The elimination half-life of the RRBC particles was calculated as 41.8 h, which is similar to the values reported for most RBC membrane fused nanoparticles,[11-12] indicating long in vivo circulation times which is potentially useful for cargo delivery functions. Following the pharmacokinetic examination, the biodistribution profile of the RRBC particles were assessed to
confirm the potential of RRBC as an in vivo delivery vehicle. The mice were sacrificed at 2, 6, 24, and 48 h post-injection of FITC-labeled RRBC particles and then the blood, liver, lung, kidney, spleen, and heart were harvested for fluorescence analysis. Figure 5.4B shows the respective RRBC particle content per gram of tissue for all the organs examined. The majority of the particles were found to be localized in the liver and spleen 24 h post-injection. This biodistribution pattern can be attributed to the erythrophagocytosis process, which removes senescent RBCs from the blood-stream and primarily takes place in the liver and spleen.\[24\text{–}25\] To further understand the overall RRBC particle distribution in each organ, the fluorescence signals were multiplied by the measured weight of the corresponding organ, assuming the weight of the blood as 7% of the total body weight. Figure 5.4C shows the relative signal in each organ normalized to the total fluorescence. After accounting for the tissue mass, the RRBC particles were found to be mainly distributed in the blood and the liver. As the blood fluorescence decreased, a corresponding increase in fluorescence signal was observed in the liver, a highly perfused organ, attributed to the abundant blood flow through the liver, and the inescapable macrophage system uptake.\[24,25\] It is worth noting that, compared to the reported particle-circulation data in mouse models,\[11,12\] for which most of the particles showed negligible blood retention after 24 h, the RRBC particles displayed significantly longer circulation time, attributed to the sufficiently low modulus and the native RBC membrane camouflage, a feature which holds tremendous potential for biomedical applications as medicament delivery and bio-detection platforms.
Figure 5.4. A) Circulation time of rebuilt RBCs (n = 3; mean ± SD). Insert table is the related elimination half-life. B) Fluorescence intensity per gram of tissue at 2, 6, 24 and 48 h after intravenous administration of rebuilt RBC (n = 3; mean ± SD). C) Relative signal per organ at 2, 6, 24 and 48 h after intravenous administration of rebuilt RBC (n = 3; mean ± SD). D) UV-Vis spectrum analysis of oxygenated and deoxygenated rebuilt RBC. Insert images show the generation of bluish glow of native and rebuilt RBCs after the addition of luminol-perborate mixture. E) Schematic illustration of the oxygen binding via hemoglobin by RBCs. F) Reversible transfer of rebuilt RBC between oxygenation and deoxygenation states. G) Time-dependent oxygenation curves of native and rebuilt RBC.

5.2.5 Oxygen carrying capability of Rebuilt RBCs

The long circulation characteristics of the RRBC particle prompted us to assess its oxygen carrying capabilities since the most important function of the native RBCs is to transport oxygen from the lungs to the body tissues with the help of the hemoglobin (Hb). In order to achieve oxygen transport, hemoglobin molecule was loaded on to the RRBC particle to yield oxygen transport-capable
material. The presence of hemoglobin was confirmed using luminol-based chemiluminescence,[4] a diagnostic tool commonly used in forensic science to detect blood stains[4] based on the iron-dependent catalysis of the reaction of the luminol-perborate with peroxide to produce a bluish glowing compound, which indicates the presence of iron-containing hemoglobin.[4] **Figure 5.4D** shows that both the native RBCs and hemoglobin loaded RRBC particles could generate bluish glow when tested with luminol in the dark, demonstrating the presence of hemoglobin. To investigate the oxygen carrying capability of the hemoglobin-loaded RRBC particles, UV-Vis absorption analysis was used to reveal the reversible shift of the maximum absorption peaks of RBCs in oxygenated and deoxygenated states,[26-28] The characteristic absorption peak of the native RBC and hemoglobin-loaded RRBC particle in oxygenated states appeared at 415 nm (**Figure 5.4D**) which after bubbling nitrogen gas for 2 h and adding reducing agent sodium dithionite (Na$_2$S$_2$O$_4$), red-shifted to 430 nm (**Figure 5.4D**), confirming the deoxygenated states of the native RBCs and the RRBC particles. These deoxygenated RRBC particles could carry oxygen after exposure to atmospheric oxygen. This process of binding and release of oxygen could be repeated several times to show that the hemoglobin-loaded RRBC particle had a similar oxygen carrying capability as the native RBCs (**Figure 5.4F**). To further investigate the oxygen carrying capability of the RRBC particles, the oxygenation rate of the deoxygenated form of RRBC was monitored by observing absorbance changes at 430 nm. Interestingly, both the native RBCs and the RRBC particles displayed a
similar oxygen curve profile (Figure 5.4G), further confirming that hemoglobin-loaded RRBC has similar oxygen carrying capability as the native RBCs.

### 5.2.6 Multifunctional Rebuilt RBCs construction

**Figure 5.5.** A) Schematic illustration of the cargo release. B) Schematic illustration of multifunctional rebuilt RBC delivery drug in tumor area. C) Schematic illustration of the manipulation of magnetic rebuilt RBC via an external magnetic field. D) Release percentage for Mn-TPPS4-loaded rebuilt RBC maintained in extracellular physiological conditions (PBS, pH 7.4) at 37°C. E) Drug release percentage for DOX-loaded rebuilt RBC maintained at 37°C. F,G) Photographs of a dispersion of magnetic RBC mimics before (F) and after (G) placing a magnet on its side. H) Bright field microscopy images of magnetically-moved magnetic rebuilt...
Due to the demonstrated ability to load RRBC particles and the long term circulation seen in \textit{in vivo} models, loading of RRBC particles with various functional cargoes non-native to RBCs to add functional dimensions was assessed. As a proof-of-concept, RRBC particles were loaded with a magnetic resonance imaging (MRI) contrast agent, 5,10,15,20-Tetrakis(4-sulfonatophenyl)-21H,23H-porphine manganese(III) chloride (Mn–TPPS4, 3.5 μg/million particles) and the anti-cancer drug, doxorubicin hydrochloride (DOX, 5.5 μg/million particles). The evidence of loading and release profiles of these cargos were examined by dialyzing loaded RRBC particles in 1x PBS and then recording the absorbance (at 412nm for Mn–TPPS4 and 485nm for DOX) of the supernatant collected at different time points to determine the quantity of the cargo released. Upon directly fusing the RBC membrane ghosts, the total Mn–TPPS4 released from the RRBC was calculated to be \( \approx 60\% \) after 40 h (\textit{Figure 5.5D}), which in the presence of an extra polymer layer between the loaded Mn–TPPS4 and the fused RBC ghost membrane, reduced to \( \approx 5\% \) of Mn–TPPS4 (\textit{Figure 5.5D}). This significant decrease in the amount of Mn–TPPS4 released was attributed to an increase in the complexity of the diffusion pathway introduced by the extra polymer layer (\textit{Figure 5.5A}). The long-term retention of Mn–TPPS4 within the RRBC after polymer coating lays supports the use of these RRBC in biomedical imaging applications.\cite{29} On the
other hand, analysis of the DOX released from the RRBC revealed greater total DOX release at pH 5.0 (~40%) compared to pH 7.4 (~10%) after 40h (Figure 5.5A and 5.5E), possibly due to the carboxylate groups on the alginate layer becoming less negatively charged at lower pH, and reducing the electrostatic interactions between the alginate layer and the DOX molecules, which should lead to increased DOX release kinetics or profiles.\[^{30,31}\] This pH dependent and slow release suggests a possible lower toxicity of DOX to normal tissues at physiological conditions versus potentially better anti-cancer activity of the DOX loaded RRBC particles in the acidic tumor micro-environment due to higher DOX release (Figure 5.5B). Besides small molecules, evidence of nanoparticle cargo loading onto the RRBC particle was also demonstrated. To prove this, magnetically active RRBC particle was fabricated by packaging RRBC with magnetic iron oxide (Fe\(_3\)O\(_4\)) nanoparticles. By controlling the external magnetic field, the ‘magnetic RRBC’ exhibited an ‘on’ and ‘off’ magnetic guidance/movements (Figure 5.5C and 5.5H), that are also potentially relevant for magnetic drug targeting, magnetic resonance imaging (MRI), magnetic hyperthermia, or the development of micromotors.\[^{32-35}\]

To further demonstrate the multifunctionality of the RRBC particle, the possible use of the RRBC as a platform for detoxification and biosensing was examined. Globally, bacterial infections are the leading cause of morbidity and mortality worldwide.\[^{36-39}\] Many of these bacteria release pore-forming toxins (PFTs) or cytotoxic proteins which are major factors in the virulence of these bacteria. PFTs form transmembrane pores on the cellular membrane, allowing uncontrolled transport of solutes across the membrane, leading to cell death via
colloid-osmotic lysis. In the design of the RRBC particle, the outer RBC-derived membrane can provide an ideal surface to absorb and neutralize PFTs, supported by the inner polymer core’s ability to stabilize the membrane and prevent PFT desorption. (Figure 5.5I) To investigate the PFT neutralization by the RRBC particles, a classical PFT, α-hemolysin (Hlα) was pre-incubated with equivalent amounts of RBC ghost membrane, RBC-polymer replica, or RRBC particles, and then mixed with 4-times equivalent of purified RBCs before hemolysis tests. Almost zero hemolytic effect was observed when Hlα was pre-incubated with the RRBC particles, relative to ~100% or ~90% RBC hemolysis from Hlα pre-incubated with the RBC ghost membrane or the RBC-polymer replica respectively, indicating that only polymer-stabilized RBC membrane in RRBC particles could prevent the hemolytic process (Figure 5.5J and S5.8). To further investigate PFT neutralization action, RBC-polymer replica or RRBC particle was mixed with the purified native RBCs (1:4 ratio), and then Hlα was added so that either of the constructs could compete with the purified RBCs for the Hlα toxin. In this competitive setup, only ~50% RBC hemolysis was observed with the RRBC particle which was much lower than the ~90% hemolysis caused by Hlα in the presence of RBC-polymer replica (Figure 5.5J), indicating that the RRBC particles were not only able to arrest the Hlα toxin effect, but also prevent its further interactions with the native RBCs.

Under normal physiological conditions, the native RBCs are characterized by high levels of adenosine triphosphate (ATP) that enable them to perform requisite physiological functions. Therefore measurement of the release of ATP
due to RBC hemolysis, represents a possible way of detecting the presence of toxins that trigger RBC hemolysis (Figure 5.5I). In order to test this concept, a luciferase-luciferin ATP biosensor was loaded within either RBC-polymer replicas or RRBCs that were then mixed with intact RBCs in PBS, lysed RBCs in PBS, or lysed RBCs in water followed by luminescence testing. Interestingly, of these systems, intact RBCs yielded no response, RBC-polymer replicas yielded a luminescent response for RBCs lysed in either PBS or water, but the biosensor loaded RRBCs yielded a luminescent response only when incubated with RBCs lysed in water (Figure 5.5K). This is explained by the fact that the biosensor loaded within the polymer replica is accessible to ATP in both water and PBS. However, the biosensor encapsulated beneath the RBC membrane in the RRBC is inaccessible in PBS and accessible only in water that osmotically lyses/permeabilizes the RRBC membrane. This suggests that the RRBC biosensor should be stable under normal physiological conditions but be activated only under conditions where RBCs are lysed producing ATP and the RRBC membrane is permeabilized. These conditions are met under conditions where PFTs (e.g. Hlα) induce RBC lysis and permeabilization of the RRBC membrane. Due to the luminescence intensity of luciferase-luciferin ATP biosensor loaded RRBCs increases linearly with increasing hemolysis content of RBCs lysis water solution, the luminescence intensity-hemolysis standard curve was determined (Figure S5.8). To evaluate applications of the RRBC biosensor based on ATP release, luciferase-luciferin ATP biosensor loaded RBC-polymer replicas or RRBCs were first pre-incubated with Hlα, and then mixed with the native RBCs.
(1:4 ratios). Notably, after pre-incubation, biosensor loaded RRBCs exhibited very low luminescence signal (~0% hemolysis), the biosensor loaded RBC-polymer replicas exhibited a 28-fold higher luminescence signal (~90% hemolysis) compared to RRBCs (Figure 5.5K), which is consistent with PFT neutralization results (Figure 5.5J). To further mimic the typical competitive binding in the body, biosensor loaded RBC-polymer replicas or RRBCs were mixed with the native RBCs (1:4 ratios) and then Hlα was added. A lower luminescence intensity was observed with the biosensor loaded RRBCs (~50% hemolysis) relative to that of the biosensor loaded RBC polymer replicas (~90% hemolysis), which matched the Hlα’s induced hemolysis observed in direct competitive setup. This study demonstrates the potential of our designed biosensor loaded RRBCs as sensors for monitoring pore-forming toxin in vivo system.

5.3 Conclusion

The construction of RRBC particles based on mimicking the unique characteristics of the native RBCs and then expanded to provide novel functionalities has been presented. The RRBC particles were constructed through three separate building blocks of silica cell bio-replication process, layer-by-layer deposition of biocompatible polymer to translate native RBCs into flexible RBC-polymer replica loadable with functional cargos to provide special functions, and encapsulation of the RBC-polymer replica loadable with the RBC derived membrane ghosts that mimic the surface properties of the native RBCs. The RRBC particle displays deformable property typical of the native RBCs, zero hemolytic activity, low cytotoxicity and vascular flow in the ex-ovo chick embryo and in vivo
mouse models. In addition, different functional cargos (e.g. hemoglobin, Mn-TPPS4, DOX, iron oxide nanoparticles and ATP biosensor) can be loaded onto the RRBC particle to enable functions such as oxygen delivery capability, MRI contrast imaging, therapeutic drug delivery, magnet-targeted localization and pore-forming toxin biosensor. Together, RRBC particle has been engineered with robust and unique features that may enable it to serve as a great tool to promote our understanding of complex life processes and possibly, be used as a good model for designing multifunctional delivery and bio-detection platforms.
5.4 Materials and Methods

5.4.1 Materials

All chemicals and reagents were used as received. Tetramethyl orthosilicate (≥99%, TMOS), sodium chloride (NaCl), hydrochloric acid (37%, HCl), Fluorescein-5-isothiocyanate (FITC), chitosan, alginate, formaldehyde (37%), glutaraldehyde solution (25% in H₂O), endothelial cell growth medium, dimethyl sulfoxide (DMSO), sodium perborate, sodium carbonate, luminol, sodium dithionite (Na₂S₂O₄), hemoglobin, doxorubicin (DOX), Mn(III)tetra (4-sulfonatophenyl) porphyrin, Iron(III) acetylacetonate [Fe(acac)₃], benzyl alcohol, α-hemolysin, luciferase and luciferin were purchased from Sigma-Aldrich. 1x- phosphate-buffered saline (1x PBS) and Blood Typing Anti-Sera, Anti-A, Anti-B and Anti Rh were purchased from Thermo Fisher Scientific. Buffered Oxide Etch (BOE) was purchased from KMG Chemicals. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (18:1 Liss Rhod PE) lipid was purchased from Avanti Lipids. Ethanol was purchased from KOPTEC. Human umbilical vein endothelium cells (HUVEC) and mouse macrophage Raw264.7 cells were obtained from the American Type Culture Collection (ATCC). Dulbecco’s modification of Eagle’s medium (DMEM) was obtained from Corning Cellgro. Fluorescein labeled Lens Culinaris Agglutinin (LCA) was purchased from Vector Laboratories. Milli-Q water with a resistivity of 18.2 MΩ cm was obtained from an inline Millipore RiOs/Origin water purification system.
5.4.2 Purification of RBCs

All the animal procedures complied with the guidelines of the University of New Mexico Institutional Animal Care and Use Committee and were conducted following institutional approval (Protocol 11-100652-T-HSC and 17-200658-HSC). Human RBCs were acquired from healthy donors with their informed consent. All blood samples were collected and stored in BD Vacutainer® blood collection tubes (Becton Dickinson, NJ, USA) containing 1.5 mg of EDTA per mL of blood for anticoagulation purposes. The purification of whole blood was carried out using Ficoll® density gradient centrifugation procedure.

5.4.3 Preparation of silica RBC replicas

Purified RBCs were fixed in 4% formaldehyde in 1x PBS at room temperature for 20h before silicification. The fixed RBCs were rinsed twice with 1x PBS, once with 154 mM NaCl solution (0.9% saline) and then suspended in a silicification solution containing 100 mM TMOS, 154 mM NaCl and 1.0 mM HCl (pH 3.0). After 24h rotation at room temperature to allow silicification process to take place, silicified RBCs were subjected to series of ethanol dehydration (30, 50, 70, 90, 100% ethanol in water) for 10 minutes each and then dried under vacuum for 24h. Dry silicified RBCs were then calcined at 500 °C for 4h in an oven by placing them in a covered (but not air tight) glass tube to generate silica RBC replicas.
5.4.4 Preparation of RBC-polymer replica

The silica RBC replicas were incubated for 2h in chitosan solution (2mg/mL in 1% acetic acid solution) under constant shaking. After rinsing with water, the particles were resuspended in alginate solution (1mg/mL in water) under constant shaking for 0.5h. Then, the particles were rinsed with water and isolated via centrifugation (1500g for 5 minutes). This process represents the typical procedure for single chitosan-alginate layer formation and it was repeated two times to achieve polymer coated silica RBC replica. In order to fabricate RBC-polymer replica, 1:10 diluted buffered oxide etch, also known as buffered HF (BOE) solution (pH~5) was used to etch the silica and yield RBC-polymer replica. [Caution! HF is highly toxic. Extreme care should be taken when handling HF solution and only small quantities should be prepared.] The RBC-polymer replica were washed with water and resuspended in double distilled water.

5.4.5. Preparation of RBC-membrane-derived ghosts

Purified RBCs were washed three times with ice cold 1x PBS, and then suspended in ice cold 0.25x PBS for 20 min to allow hemolysis to take place. After treatment with hypotonic solution (0.25x PBS), the released hemoglobin was removed via centrifugation (1000g for 5 minutes), whereas the pellet (RBC ghost) with light pink color was collected and washed twice with 1x PBS. The RBC ghosts (devoid of cytoplasmic contents) were mixed with 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (18:1 Liss Rhod PE) and verified under fluorescence microscope, which revealed a hollow spherical
structure of RBC membrane ghosts.

5.4.6 Preparation of rebuilt RBC (RRBC) particles

To prepare the RRBC particles, the RBC membrane ghosts were fused onto RBC-polymer replica. Briefly, $4 \times 10^7$ RBC-polymer replica quantified by hemocytometer were mixed with RBC membrane ghosts prepared from $1 \times 10^8$ purified RBCs and then sonicated in ice cold water bath for 1 min. An excess of RBCs was used to compensate for the membrane loss during RBC ghost derivation. The resulting RRBC particles were carefully centrifuged (5000g for 10 minutes) and the excess membrane components remaining in the supernatant were removed.

5.4.7 Zeta potential measurements

Zeta potential measurements were made using Malvern Zetasizer Nano-ZS (Westborough, MA, USA) equipped with a He–Ne laser (633 nm) and non-invasive backscatter optics (NIBS). The layer-by-layer samples, silica RBC replica, RBC-polymer replica, and RRBC particles for zeta potential measurements were suspended in 5 mM NaCl solution, while the zeta potential measurements for the RBC and RRBC particles was acquired in 154 mM NaCl solution (0.9% saline) using monomodal analysis tool. All reported values correspond to the average of at least three independent samples.

5.4.8 Scanning electron microscopy (SEM) imaging

The morphology of fixed RBC, silica RBC replica, polymer coated silica
RBC replica and RBC-polymer replica samples were characterized using scanning electron microscope (SEM). SEM samples were prepared by drop casting. Briefly, all samples were suspended in water, and then dropped onto 5×5 mm glass slides. The glass slides were then mounted on SEM stubs using conductive adhesive tape (12 mm OD PELCO Tabs). Samples were sputter coated with a 10 nm layer of gold using a Plasma Sciences CrC-150 Sputtering System (Torr International, Inc.). SEM images were acquired under high vacuum, at 10 kV, using an FEI Quanta series scanning electron microscope (Thermo Fisher Scientific, MA, USA).

5.4.9 Confocal microscopy imaging

RRBC particles were spotted onto glass cover slips. Slides were mounted using Vectashield Antifade. Confocal images were acquired with a 63X/1.4NA oil objective in sequential scanning mode using a Leica TCS SP8 confocal microscope.

5.4.10 Microfluidic blood capillary model and experiments

The microfluidic blood capillary model mimicking dimensions (5 μm in diameter and 50 μm in length) and pressure drops of human blood capillaries was prepared as described previously. In brief, a master pattern was designed using computer-aided design software (AutoCAD 2013, Autodesk, USA) and then simulation software (COMSOL Multiphysics 4.3, USA) was used to refine and validate the design. The designed master pattern was then transferred to the silicon wafer using chrome mask and negative photoresist and then transferred into polydimethylsiloxane (PDMS) to achieve microfluidic blood capillary device.
through soft lithography. The microfluidic experiments were then performed as follows. The non-sample reservoirs were filled with 1x PBS, and the device was then connected to a pressure controller (NE-300, New Era Pump System) and placed on top of a Zeiss AxioExaminer upright microscope. For the microfluidic tests, 1x10⁶/mL particles (RBCs, silica RBC replicas, and RRBC particles cross-linked with different cross-linker concentrations) in PBS were assessed for deformability characteristics. The different pressures used for the two inlets were chosen based on the finite element simulations to achieve physiologically relevant pressure drops over the microchannels (Supplementary Table 1).[1]

5.4.11 Antibody-mediated agglutination assay

Briefly, 1x10⁶ native RBCs or RRBC particles were suspended in 450μL of 1x PBS (pH 7.4) solution, and then 50μL of anti-type sera [anti-A, anti-B, and anti-D (Rh)] were added. After 15min, the bright field images were acquired on the Leica DMI3000 B inverted microscope to evaluate occurrence of agglutination or lack thereof.

5.4.12 Immunofluorescence staining

The native RBC and RRBC particles were blocked with 5% BSA in 1x PBS, and then incubated with fluorescent antibodies against ICAM-4 (R&D Systems) and CD47 (Biolegend) proteins for 30min. The samples were then rinsed with 1x PBS, and then suspended again in 1x PBS. Microscopy images were then obtained on the Leica DMI3000 B inverted fluorescence microscope.
5.4.13 Hemolysis assay

Purified RBCs were incubated with different concentrations of silica RBC replicas, polymer coated silica RBC replicas, RBC-polymer replica, and RRBC particles at 37°C for 2h in continuous rotating state. Double distilled (D.I.) water and 1x PBS containing purified RBCs were used as the positive and negative controls, respectively. The absorbance of hemoglobin in the supernatant was measured by a BioTek microplate reader (Winooski, VT) at 540nm. The hemolysis percentage of each sample was determined using the reported equation \[^{3,4}\] as;

\[
\text{Percent hemolysis} \, (\%) = 100\times\frac{(\text{Sample Abs}_{540\text{nm}}-\text{Negative control Abs}_{540\text{nm}})}{(\text{Positive control Abs}_{540\text{nm}}-\text{Negative control Abs}_{540\text{nm}})}
\]

5.4.14 Cell viability assay

Cell culture was performed using standard procedures. HUVEC and Raw264.7 cells were maintained in the respective media of endothelial cell growth medium and DMEM containing 10% FBS at 37 °C and 5% CO\(_2\). Cells were passaged at approximately 70% confluency. For cell viability assays, 100 μL of cell suspension (100,000 cells/mL) were seeded into a 96-well plate (White Opaque) and cultured for 24h at 37 °C. The cells were then incubated with 100 μL of different concentrations of silica RBC replicas, polymer coated silica RBC replicas, RBC-polymer replica, and RRBC particles solutions. After 24h incubation, 100 μL of CellTiter-Glo 2.0 Reagent was added into each well and incubated for 10 min at room temperature. The luminescence readings were then obtained/recorded using BioTek microplate reader. The percent cell viability was calculated relative to the
control non-treated cells.

5.4.15 Test of vascular flow in ex ovo chick embryos

The vascular flow characteristics of RRBC particles were tested using Ex ovo chick embryo model as described previously[3] and was conducted following institutional approval (Protocol 11-100652-T-HSC). Briefly, eggs were acquired from East Mountain Hatchery (Edgewood, NM) and placed in a GQF 1500 Digital Professional incubator (Savannah, GA) for 3-4 days. Embryos were then removed from shells by cracking into 100ml polystyrene weigh boats. Ex ovo chick embryos were covered and incubated at 37°C, 70% humidity. 50μL (at 4 x 10^7 particles/mL) of samples (RBC-polymer replica, silica RBC replica@polymer-RBC ghost [silica-RRBC], and RRBC particles) in 1x PBS were injected into the secondary or tertiary veins via pulled glass capillary needles and then, the CAM vasculature was imaged using a customized avian embryo chamber and a Zeiss Axio Examiner upright microscope with heated stage.

5.4.16 Pharmacokinetics and biodistribution studies

All the animal procedures complied with the guidelines of the University of New Mexico Institutional Animal Care and Use Committee and were conducted following institutional approval (Protocol 17-200658-HSC). The experiments were performed on female Albino C57BL/6 mice (6 weeks) from Charles River Laboratories. To evaluate the circulation half-life of RRBC particles, 100 μL of FITC-labeled RRBC particles (3x10^7 particles/mL) were administered by retro-orbital injections. Blood was collected at 2, 6, 24, and 48h post-injection with each
group contained three mice. Blood samples were diluted 1:1 with PBS prior to fluorescence measurements. Particle retention in circulation was determined by measuring the fluorescence using a BioTek microplate reader (Winooski, VT). Pharmacokinetics parameters were calculated to fit a two-compartment model. To calculate the elimination half-life, the normalized signal was transformed using natural log, and the elimination phase from the resulting data was fitted using a linear regression curve. Elimination half-life was calculated as  \( t_{1/2} = \ln(2)/\beta \), where \( \beta \) is the negative slope obtained from the fit. The value of \( \beta \) with standard error for the RRBC particles was 0.01655 ± 0.0028.

To study the biodistribution of the RRBC particles in various tissues, similar to the preceding study, 100 μL of FITC-labeled RRBC particles were administered by retro-orbital injection. At 2, 6, 24, and 48h following the particle injection, three mice were randomly selected and euthanized. Their blood, liver, lung, kidney, spleen, and heart were collected. The collected organs were weighed and then homogenized in 1 mL PBS. Total weight of blood was estimated as 7% of mouse body weight. The fluorescence intensity of each sample was determined by a BioTek microplate reader (Winooski, VT).

5.4.17 Hemoglobin loading

The chitosan surface of the RBC-polymer replica was incubated for 24h with 5mg/mL hemoglobin under constant shaking at 4 °C. After rinsing with water, the particles were resuspended in 2mg/mL chitosan solution under constant shaking for 0.5h at 4 °C. This process was repeated three times to achieve enough
hemoglobin loading. The particles were then resuspended in 1mg/mL alginate solution under constant shaking for 0.5h and then the RBC membrane ghosts were fused onto hemoglobin loaded RBC-polymer replica to generate hemoglobin loaded RRBC particles.

5.4.18 Chemiluminescence assays

The luminol-based method for evaluating oxygen carrying ability of the RRBC particles was adopted from Doshi, Nishit, et al. [5] Briefly, 70mg sodium perborate, 500mg sodium carbonate, and 200mg luminol were added to 5 mL water and dissolved with sonication to achieve luminol solution. The luminol solution was left undisturbed for 5 min in a dark room. For imaging purposes, 1mL of luminol solution was added to 4mL samples (5million native RBCs and RRBC particles) in 1x PBS (pH 7.4) solution. The optical image was taken by Sony ILCE-5100 Camera (ISO-100 and exposure time 1/15s). The chemiluminescence optical image was taken in a dark room by Sony ILCE-5100 Camera (ISO-6400 and exposure time 30s). For luminescence assay, 100μL of samples (5million native RBCs and RRBC particles) in 1x PBS (pH 7.4) solution were added into white 96-well plates at a density of 5million cells/mL. After that, 20μL of luminol solution was added to each well. Mix the contents for 2min on shaker in the dark. Luminescence was measured using a BioTek microplate reader. The luminescence was expressed as a relative percentage of the control.

5.4.19 Assay of the reversible binding of oxygen

The ability of the RRBC particle to reversibly binding oxygen was detected
by analyzing changes of UV–Vis absorption spectrum (300–700 nm) in oxygenated and deoxygenated solutions. For complete deoxygenation, nitrogen gas was bubbled into the sample solution to displace oxygen. After 2h, sodium dithionite (Na₂S₂O₄) was added, and UV–Vis absorption spectrum was obtained by a BioTek microplate reader. For oxygenation, sample solutions were exposed to atmospheric oxygen for more than 2h, and then UV–Vis absorption spectrum was recorded as before. This process represents the typical procedure used to test reversible oxygen binding capability and it was repeated two times. The deoxygenated sample (λ_{max} = 430 nm) could be gradually converted to oxygenated sample (λ_{max} = 415 nm) by exposing it to air atmosphere at room temperature. The oxygenation rate of the deoxygenated sample was monitored by observing changes in absorbance via UV–Vis spectroscopy. The oxygenation state of each sample was calculated using the following equation: Oxygenation state (%) = 100*(Abs_{t0} - Abs_{t})/(Abs_{t0} - Abs_{tmin}) where Abs_{t0} and Abs_{t} represent the 430 nm absorbance at the starting point (t = 0, complete deoxygenated state) and at the specific time, respectively, and Abs_{tmin} represents the 430 nm absorbance at the minimum value.

5.4.20 Loading and release kinetics of small molecules

In order to load the negatively charged Mn–TPPS4, the chitosan surface of the RBC-polymer replica was incubated with Mn–TPPS4 (2mg/mL) for 4h under constant shaking. RBC membrane ghosts were then directly fused onto the Mn–TPPS4 loaded RBC-polymer replica. Note that for the extra polymer layer samples, the particles were resuspended in 1mg/mL alginate solution under constant
shaking for 0.5h and then the RBC membrane ghosts were fused onto the Mn–TPPS4 loaded RBC-polymer replica. For the positively charged doxorubicin (DOX) loading, the alginate surface of the RBC-polymer replica was incubated for 4h in 3mg/mL DOX under constant shaking. Then, the particles were resuspended in 1mg/mL alginate solution under constant shaking for 5min followed by the fusion of the RBC membrane ghosts onto the DOX loaded RBC-polymer replica. To quantify loading of Mn–TPPS4 and DOX, microplate reader UV–Vis measurements were obtained at 410nm for Mn–TPPS4 and 475nm for DOX. The Mn–TPPS4 loading capacity was found to be 3.5 μg/million particles while the DOX loading capacity was 5.5 μg/million particles. The dialysis bag diffusion method was used to evaluate Mn–TPPS4 and DOX release kinetics. Briefly, particles were loaded into 20kDa MWCO Por Float-A-Lyzer G2 dialysis device, sealed in 50 mL conical tubes containing 20mL phosphate-buffered saline (pH 7.4 or 5), and kept at 37 °C while stirring. At definite time points, 1 mL of dialysate was removed for absorbance analysis on a BioTek microplate reader and then 1 mL of the fresh dialysate solution was added to the conical tube. Each batch of experiments was performed in triplicate.

5.4.21 Magnetic iron oxide nanoparticles synthesis

Bare magnetic iron oxide (Fe₃O₄) nanoparticles were synthesized according to the previous method. Briefly, 0.687 g of Fe(acac)₃ (1.94 mmol) was dissolved in 9 mL of benzyl alcohol. The solution was heated to 170 °C under reflux and stirring at 1500 rpm for 24 h. After the reaction was cooled down to room temperature, 35 mL of EtOH was added into the mixture, and then centrifuged at
20000 rpm for 10 min. The supernatant was discarded, and the resulting precipitate was washed with EtOH twice to yield the required Fe₃O₄ nanoparticles. The synthesized Fe₃O₄ nanoparticles were stored in EtOH before use.

**5.4.22 Loading of magnetic nanoparticles**

The bare Fe₃O₄ nanoparticles (NP) were incubated in 1mg/mL chitosan solution overnight and then washed with DI water twice prior to the actual loading experiments. Chitosan coated Fe₃O₄ nanoparticles were then incubated with the alginate surface of the RBC-polymer replica for several hours under constant shaking followed by another resuspension in alginate solution (1mg/mL) under constant shaking for 0.5h to ensure optimal display of the alginate’s negative charge. The RBC membrane ghosts were then fused onto the Fe₃O₄ loaded polyion to generate Fe₃O₄ loaded RRBC particles.

**5.4.23 Confirmation of the Fe₃O₄ nanoparticle loading**

The magnetic Fe₃O₄ nanoparticle loaded RRBC particles were suspended in an external magnetic field produced by a neodymium magnet. The bright field images were then obtained on the Leica DMI3000 B inverted microscope to evaluate the magnetic guidance response.

**5.4.24 Quantification of toxin hemolytic activity**

Briefly, 1.5x10⁷ purified RBCs were incubated with 1mL of different concentrations of α-hemolysin in PBS at 37°C for 30min. D.I. water and 1x PBS containing purified RBCs were used as the positive and negative controls,
respectively. The absorbance of hemoglobin in the supernatant was measured by a BioTek microplate reader (Winooski, VT) at 540nm. The hemolysis percentage of each sample was determined using the reported equation \[^{[3,4]}\] as; Percent hemolysis (\%) = 100*(Sample Abs\(_{540\text{nm}}\) - Negative control Abs\(_{540\text{nm}}\))/(Positive control Abs\(_{540\text{nm}}\) - Negative control Abs\(_{540\text{nm}}\))

5.4.25 Toxin absorption study \textit{in vitro}

Briefly, 3x10\(^6\) RRBC particles were incubated with 1mL of different concentrations of \(\alpha\)-hemolysin in PBS at 37\(^\circ\)C for 30min., followed by addition of 1.2x10\(^7\) purified RBCs for additional 30 min incubation. The hemolysis percentage of each sample was determined using the absorbance of hemoglobin in the supernatant.

5.4.26 Toxin neutralization \textit{in vitro}

In vitro toxin neutralization ability of RRBC particles was examined in two parts. In part 1, 1mL\(\alpha\)-hemolysin (20\(\mu\)g/mL in PBS) was incubated with RBC ghost membrane (from 7.5x10\(^6\) RBCs), 3x10\(^6\) RBC polymer replicas, and 3x10\(^6\) RRBC particles at 37\(^\circ\)C for 30mins. After this incubation, 1.2x10\(^7\) purified RBCs were added into the respective formulations. The mixture was incubated for an additional 30 min at 37\(^\circ\)C prior to hemolysis quantification. In part 2, 1mL\(\alpha\)-hemolysin (20\(\mu\)g/mL in PBS) was directly incubated with the mixture of 3x10\(^6\) RRBC particles and 1.2x10\(^7\) purified RBCs at 37\(^\circ\)C for 30mins, followed by hemolysis quantification. The hemolysis percentage of each sample was determined using the absorbance of hemoglobin in the supernatant.
5.4.27 Luciferase-luciferin biosensor loading

The alginate surface of the RBC-polymer replica was incubated for 24h with 3mg/mL luciferin under constant shaking in the dark. After rinsing with water, the particles were resuspended in 1mg/mL alginate solution for 10min, and then incubated in 2mg/mL chitosan solution for 30min. For the luciferase loading, the chitosan surface of the luciferin loaded RBC-polymer replica was incubated overnight in 1mg/mL luciferase in PBS under constant shaking at 4 °C in the dark. Then, the particles were resuspended in 2mg/mL chitosan solution under constant shaking for 5min, followed by 1mg/mL alginate solution for 10min. In order to have more luciferin, 3mg/mL luciferin was added to luciferase-luciferin loaded RBC-polymer replica again and incubated for 4h under constant shaking at 4 °C in the dark. After rinsing with PBS, the particles were then resuspended in 1mg/mL alginate solution under constant shaking for 5min and then the RBC membrane ghosts were fused onto luciferase-luciferin biosensor loaded RBC-polymer replica to generate luciferase-luciferin biosensor loaded RRBC particles.

5.4.28 Luciferase activity assay

Since the goal was to test the ATP released from the RBCs, the biological activity of luciferase was measured using different concentrations of water lysed RBC solution. In brief, a solution containing 1.2x10⁶, 3x10⁶, 6x10⁶, 9x10⁶, and 1.2x10⁷ lysed RBCs in water (10%, 25%, 50%, 75%, 100% hemolysis, respectively) was added to a solution of 3x10⁶ luciferase-luciferin biosensor loaded RRBC particles and MgSO₄ (8 mM) in a final volume of 1mL. Luminescence intensity was
then measured immediately by a BioTek microplate reader (Winooski, VT).

5.4.29 Evaluation of Pore-forming toxin sensor

The Pore-forming toxin sensor property of RRBC particles was assessed in three parts. In the first part, 1mL PBS or D.I. water, and toxin (20μg/mL in PBS) was incubated with a mixture of $3 \times 10^6$ luciferase-luciferin biosensor loaded RRBC particles or luciferase-luciferin biosensor loaded RBC polymer replicas and $1.2 \times 10^7$ purified RBCs at 37°C for 15mins, followed by luminescence measurements. In the second part, 1mL of $1.2 \times 10^7$ lysed RBCs in PBS was incubated with $3 \times 10^6$ luciferase-luciferin biosensor loaded RRBC particles or luciferase-luciferin biosensor loaded RBC polymer replicas at 37°C for 15mins, respectively, followed by luminescence measurements. These lysed RBCs in PBS were generated through bath sonicated disruption of RBCs. In the third part, 1mL α-hemolysin (20μg/mL in PBS) was incubated with $3 \times 10^6$ luciferase-luciferin biosensor loaded RRBC particles or luciferase-luciferin biosensor loaded RBC polymer replicas at 37°C for 15mins, and then $1.2 \times 10^7$ purified RBCs were added into the respective formulations. The mixture was incubated for an additional 15 min at 37 °C prior to luminescence quantification. Luminescence intensity was measured immediately by a BioTek microplate reader (Winooski, VT).
5.5 References


CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS
6.1 Conclusions

With all its complexities and different biofunctionalities, a cell is the basic structural and functional unit of all living organisms with the exception of viruses. My thesis work focused on functional modification of mammalian cells using silicification and modular nanoparticle (NP) exoskeletons as well as the building of a multifunctional artificial RBC system.

In Chapter One, I briefly reviewed the development and challenges of whole cell bioactivities preservation, cell in shell modification and artificial cell construction.

In Chapter Two, we proved that silica cell replication method preserves biofunctionalities of proteins in a silicified cell and maintains them in a “lifelike” state for a long period of time. It is shown that we can use antibody and nanoparticle probes to determine the accessibility and preservation of these biomarkers displayed on both the extracellular membrane and intracellular compartments throughout the course of silicification, dehydration, and desilicification. It is also shown that the activities of esterase, phosphatase (acid and alkali), and protease enzymes in hybrid silica biocomposites are preserved both in the short and long-term. Here we expect our silicified cells with preserved biofunctionality could serve as a tumor-associated antigen presentation platform for autologous cancer cell vaccine and a robust and stable whole cell bioreactor for industrial cascade enzyme catalysis.
In Chapter Three, we have developed a general, simple, and modular approach to create a new class of living hybrid materials termed SupraCells with diverse possible functionalities. Using linker chemistries we encapsulate mammalian cells within nanoparticle-based exoskeletons in an instantaneous process that avoids/abolishes NP internalization pathways such as phagocytosis. The NP exoskeletons are shown to be continuous and to maintain cell viabilities for a long period of time in a non-replicative state endowed with extremophile-like properties. Metal chelation disrupts the linker chemistry and cells return to their native states. The exoskeletons exhibit size selective permeability protecting the cells against toxins and pathogens exceeding 5-nm in diameter. Potential SupraCell functionalities are as diverse as the NP exoskeleton building blocks themselves. SupraCells demonstrated high viabilities with preserved typical metabolic signatures of the native cells, enhanced resistances against both endogenous and exogenous stressors, and extraordinary properties foreign to native cells based on the nature of the nanobuilding blocks integrated into their unique exoskeletons. We envision that this modular coating strategy will open new avenues to simple manufacturing pathways of complex micro- and/or macroscale hybrid mammalian cell-based functional materials and devices.

In Chapter Four, we have developed a general, simple, and modular approach to create a novel class of hybrid biomaterials termed Armored RBCs with diverse possible functionalities. Using metal-phenolic chemistry we encapsulated native RBCs with MOF NP-based exoskeletons in seconds without RBC lysis. The modularity and simplicity of this method arises from fast MOF NPs super-assembly
at the RBC membrane surface and enables the transformation of different MOF building blocks and RBC vehicles into diverse functional hierarchitectures. The presented Armored RBCs preserve the original properties of native RBCs, show enhanced resistances against external stressors, and exhibit extraordinary new properties that are foreign to native RBCs based on the highly modular nature of MOF nanobuilding blocks integrated into the RBC exoskeletons. The presented approach profits from the wide range of variable MOF NPs and opens the door to design of multimodal nano-superstructures for multimodal imaging, image-guided therapies and theranostics. The strategy of Armored RBCs, however, is not restricted to red blood cells alone, but can be further extended to any other cell types like leucocytes playing a pivotal role in immunity and inflammatory processes. We believe our findings will open new avenues for artificially designed cell-inspired functional materials for wide ranging biomedical applications.

In Chapter Five, we have presented a pathway to construction synthetic RRBCs that mimic the unique characteristics of native RBCs and expand upon them to provide novel functionalities. The RRBC particles were constructed by three stages of assembly: silica cell bio-replication of native RBCs, layer-by-layer deposition of a biocompatible polymer to translate the native RBC shape into a flexible RBC-polymer replica loadable with functional cargos, and encapsulation of the functionalized RBC-polymer within a RBC derived membrane ghost that confers to the RRBC the surface properties of the native RBCs. The RRBC particle displays deformability typical of native RBCs, zero hemolytic activity, low cytotoxicity and sustained vascular flow in the ex-ovo chick embryo and in in vivo
mouse models. In addition, different functional cargos (e.g. hemoglobin, Mn-TPPS4, DOX, iron oxide nanoparticles and ATP biosensor) can be loaded onto the RRBC particle to enable functions such as oxygen delivery capability, MRI contrast imaging, therapeutic drug delivery, magnetic field directed movement and localization, and pore-forming toxin biosensing. Together, RRBCs have been engineered with robust and unique features that may enable them to serve as a new tool to promote our understanding of complex life processes and possibly as a new multifunctional delivery and bio-detection platform.

6.2 Future direction

Since we have already proved that silica cell replication method preserves biofunctionalities of proteins in a silicified cell and maintains them in a "lifelike" state for a long period of time. In the future, I will generate autologous cancer vaccines using silicified cancer cells that retain innate structural features and take on additional properties, including enhanced stability and high surface absorptivity. Binding of pathogen-associated molecular patterns (PAMPs) to the silicified cell surface transforms cancer cells in pathogen mimics, able to harness and guide immune responses of cancer therapy.

The cross-linked NP-exoskeletons can provide cytoprotection of the encased cell against external stressors as well as introduce multi-functional units with intelligent features. As a consequence of encapsulation, one can surmise that the supracell deploys different coping mechanisms to deal with the stress induced by the encapsulation. Thus, I hypothesize that NP encapsulation enables a
supracell to assume a pseudo hibernation state which upregulates the autophagic pathway as a cellular coping mechanism or response’. This hypothesis will be examined by the levels of LC3 protein (known autophagic marker) in the supracell in comparison to that of the control cells not covered by the NPs.

RRBCs have been engineered with robust and unique features that may enable them to serve as a great multifunctional delivery and bio-detection platform. In Chapter Five, we have already built luciferase-luciferin ATP biosensor loaded RRBCs as sensors for monitoring pore-forming toxin in vitro. In the future, I will continue this work and show it could be utilized not only in vivo but also in vivo system.
SUMMARY OF CONTRIBUTIONS

For the work detailed in Chapter 1, I performed the majority of the literature research, writing, and revisions of this review, and Dr. Jacob Agola and Dr. C. Jeffrey Brinker assisted in manuscript writing and review.

For the work detailed in Chapter 2, I performed the majority of this research at the University of New Mexico in the Brinker lab. I wish to acknowledge contributions made by the following individuals. The original silicification concept was conceived of by Dr. Bryan Kaehr and Dr. C. Jeffrey Brinker, and engineered by Dr. Bryan Kaehr and Dr. Jason Townson for preserving the morphological features of complex biological systems, and further developed by myself for bioactivities preservation. Dr. Jacob Agola provided me with mentorship and guidance for bioactivities detection technology, contributed many ideas through helpful scientific discussions, and careful review and editing of the manuscript. Dr. Wei Zhu assisted in several electron microscopy images and provided helpful ideas through discussions, and provided careful review of the manuscript.

The work detailed in Chapter 3 was submitted as a communication article, "SupraCells: Living Mammalian Cells Protected within Functional Modular Nanoparticle-Based Exoskeletons", in the scientific journal Advanced Materials. As the primary author of this article, I performed the majority of this research at the University of New Mexico in the Brinker lab. I wish to acknowledge contributions made by the following individuals. Dr. Wei Zhu provided me with mentorship and guidance for particle synthesis technology, assisted in many of the characterization
techniques, contributed many ideas through helpful scientific discussions, and careful review and editing of the manuscript. Dr. Jacob Agola assisted in several of the ex vitro experiments, provided ideas through scientific discussions, and provided careful review of the manuscript. Dr. Shahrouz Amini assisted in mechanical test technology and provided nano-indentation support for the manuscript. Dr. Andreas Zimpel and Dr. Stefan Wuttke assisted in several nanoparticle synthesis, and careful review and editing of the manuscript. Dr. Achraf Noureddine assisted in several nanoparticle synthesis. Dr. Jonas Croissant, Dr. Yi Ju, Dr. Jin Shang, and Dr. Frank Caruso provided helpful ideas through discussions, and provided careful review of the manuscript.

The work detailed in Chapter 4 will be submitted as a communication article, "Armored red blood cells: multifunction integration through modular super-assembly of hybrid nanoparticle-based building blocks". As the primary author of this article, I performed the majority of this research at the University of New Mexico in the Brinker. I wish to acknowledge contributions made by the following individuals. Dr. Wei Zhu provided me with mentorship and guidance for particle synthesis technology, assisted in many of the characterization techniques, contributed many ideas through helpful scientific discussions, and careful review and editing of the manuscript. Dr. Rita Serda assisted in circulation study in vivo mice model and provided support for the manuscript. Dr. Evelyn Ploetz and Dr. Stefan Wuttke assisted in several nanoparticle synthesis, provided helpful ideas through discussions, and careful review and editing of the manuscript. Dr. Achraf
Noureddine assisted in several nanoparticle synthesis. Dr. Jacob Agola provided careful review of the manuscript.

The work detailed in Chapter 5, will be submitted as a communication article, "Biomimetic Rebuilding of Multifunctional Red Blood Cell: Design Using Functional Modules". As the primary author of this article, I performed the majority of this research at the University of New Mexico in the Brinker. I wish to acknowledge contributions made by the following individuals. Dr. Jacob Agola provided me with mentorship, contributed many ideas through helpful scientific discussions, and careful review and editing of the manuscript. Dr. Wei Zhu assisted in many of the characterization techniques and provided helpful ideas through discussions, and provided careful review of the manuscript. Dr. Rita Serda assisted in circulation study in vivo mice model and provided support for the manuscript. Dr. Kimberly Butler assisted in several of the ex ovo experiments, and assisted in the writing and review of this manuscript. Joshua Minster helped to synthesize rebuilt RBC. Dr. Jonas Croissant provided careful review of the manuscript.

Dr. C. Jeffrey Brinker provided financial, laboratory, and material support, mentorship, assisted in the writing, review, and approval of the manuscript. In addition, Dr. Brinker provided guidance and overall advice on the project development and direction.
APPENDIX 1
1.1 Supporting Figures

**Figure S2.1.** Optical image of fresh silicification solution and after storage 3 week.

**Figure S2.2.** A) Schematic of $^{29}$Si CPMAS NMR sample preparation. B) Schematic of HMDS quenching. C) $^{29}$Si CPMAS NMR spectra of silicified cells after different silicification time. D) Deconvolution of $^{29}$Si CPMAS NMR spectra.
Figure S2.3. Confocal microscopy images of A549 cell incubated with EGFR targeted nanoparticles (green) and Hoechst (blue) during silicification and desilicification process.

Figure S2.4. Confocal microscopy images of A549 cell plasma membrane with Anti-EGFR (magenta), EGF ligand (red), and Hoechst (blue) during silicification process.
Appendix 2 was adapted from
Wei Zhu‡, Jimin Guo‡, Shahrouz Amini, Yi Ju, Jacob Ongudi Agola, Andreas Zimpel, Jin Shang, Achraf Noureddine, Frank Caruso, Stefan Wuttke, Jonas G. Croissant*, C. Jeffrey Brinker* SupraCells: Living Mammalian Cells Protected within Functional Modular Nanoparticle-Based Exoskeletons. Submitted to Advanced Materials. (‡Authors contributed equally to this work)
2.1 Supporting Figures

**Figure S3.1.** Optical image of the pellets of HeLa cells, Supra-HeLa cell-MIL-100 (Fe), and Supra-HeLa cell-ZIF-8.

**Figure S3.2.** Fourier transform infrared spectrophotometry (FT-IR) of native HeLa cells, tannic acid, ZIF-8 NPs, and Supra-HeLa cell-ZIF-8.
Figure S3.3. SEM image of a Supra-HeLa cell-ZIF-8.

Figure S3.4. EDS carbon, oxygen, and zinc elemental mappings of the Supra-HeLa cell-ZIF-8.
**Figure S3.5.** Bright field images of A549 cell (A) and Supra-A549 Cell-ZIF-8 (B).

**Figure S3.6.** Bright field images of HL-60 cell (A) and Supra-HL-600 cell-ZIF-8 (B).
Figure S3.7. TEM (A) and SEM image (B) of ZIF-8 NPs. (C) Wide PXRD patterns of the simulated ZIF-8 and as-synthesized ZIF-8. (D) DLS data of the as-synthesized ZIF-8 NPs in water or EtOH.
Figure S3.8. TEM (A) and SEM image (B) of MIL-100(Fe) NPs. (C) Wide PXRD patterns of the simulated MIL-100(Fe), and as-synthesized MIL-100(Fe). (D) DLS data of the as-synthesized MIL-100(Fe) NPs in water or EtOH.
Figure S3.9. TEM (A) and SEM (B) image of UiO66-NH$_2$ MOF NPs. (C) Wide PXRD patterns of the simulated UiO66-NH$_2$ and as-synthesized UiO66-NH$_2$. (D) DLS data of the as-synthesized UiO66-NH$_2$ in water or EtOH.
Figure S3.10. TEM (A-B) and SEM (C) images of amine-functionalized mesoporous silica NPs; DLS data of amine-functionalized mesoporous silica NPs in water or EtOH.
Figure S3.11. TEM image of Fe₃O₄ NPs

Scheme S3.1. Schematic illustration of the fabrication of SupraCells.
Fig. S3.12. The molecular structure of 4-arm-PEG5K-SH.

Figure S3.13. SEM images of the Supra-HeLa cell-MIL-100 (Fe) (A), Supra- Raw 264.7 cell-UiO-66-NH₂ (B), and Supra-HeLa cell-MET-3(Fe) (C).
Figure S3.14. (A) TEM image tannic-modified mesoporous silica NPs. (B-C) SEM image of Supra-HeLa cell-mSiO₂. (D-F) Fluorescence image of Supra-HeLa cell-mSiO₂: nucleus, mSiO₂ exoskeleton, combined image (from left to right).
**Figure S3.15.** (A) TEM image thiol-modified mesoporous silica NPs. (B) SEM image of Supra-HeLa cell-mSiO$_2$. (D-F) Fluorescence image of Supra-HeLa cell-mSiO$_2$: nucleus, mSiO$_2$ exoskeleton, combined image (from left to right).
Figure S3.16. Optical (left) and SEM image (right) of magnetic Supra-HeLa cell-$\text{Fe}_3\text{O}_4$. 
Figure S3.17. Fluorescence microscopy images of mSiO$_2$ (red) (A-C) and UiO-66-NH$_2$ (green) (D-F) NPs internalized by HeLa cells at different time intervals of 5 min (A and D), 1 h (B and E), and 6 h (C and F).
Figure S3.18. Fluorescence microscopy images of mSiO$_2$ (cyan) internalized by HeLa cells with or without ZIF-8 NP-based exoskeletons at different time intervals of 0 h (A,D), 1 h (B,E), and 5 h (C,F). At the time of 0 h, the HeLa cells were pre-incubated with mSiO$_2$ (red) for 5 h.
Figure S3.19. Cell viability of various SupraCells after NPs coating.
Figure S3.20. Optical microscopy images of native HeLa cells (A) and Supra-HeLa cell-ZIF-8 (B) in culture flasks after cell seeding and 24 h culture.
Figure S3.21. Cell viability of Supra-HeLa cell-ZIF-8/MIL-100(Fe) after the treatment of EDTA for 30 min to remove the MOF shell.
Figure S3.22. Supra-HeLa cell-ZIF-8 attachment and proliferation after the removal of ZIF-8 shell at the encapsulation time of 2 h (A) and 24 h (B).
Figure S3.23. The proliferation of native HeLa cell (A-C) and Supra-HeLa cell-ZIF-8 after shell removal after the encapsulation for 24 h (D-F) at different time intervals: 6 h, 24 h, and 72 h.
Figure S3.24. The cell proliferation rate of native HeLa cell and Supra-HeLa cell-ZIF-8 after shell removal after the encapsulation for 24 h.
Figure S3.25. Confocal image of HeLa cell in PBS (1X) solution (A) and Supra-HeLa cell-MIL-100(Fe) in different PBS solution: 1X (B), 0.25X (C), and 5X (D).
Figure S3.26. Cell viability of native HeLa cell and Supra-HeLa cell-MIL-100(Fe) under UV irradiation (254 nm, 4W) for different incubation times.
Figure S3.27. UV-Vis spectra of Congo red and SupraCell-MIL-100(Fe) based on HeLa cells with or without Congo red dye loading in PBS (1X) solution.
Figure S3.28. Cell viability of native HeLa cell and Supra-HeLa cell-MIL-100(Fe) under UV irradiation (365 nm, 4W) for different incubation times.
Figure S3.29. Loading-unloading curves for native HeLa cells and Supra-HeLa cell-ZIF-8 and Supra-HeLa cell MIL-100(Fe) with different coating thicknesses.
Figure S3.30. Stiffness/elastic modulus versus contact depths for Supra-HeLa cell-ZIF-8 and Supra-HeLa cell-MIL-100(Fe) with different coating cycles (Z1-Z3 or M1-M3 means the coating cycles of 1 to 3).
Figure S3.31. Schematic illustration of the two mesocages in MIL-100(Fe)

Figure S3.32. Size-selective permeability studies of SupraCell-MIL-100(Fe) involving drug (DOX) permeation based on cell viability study.
**Figure S3.33.** Fluorescent emission spectra of mesoporous silica NPs functionalized with different fluorescent dyes.

**Figure S3.34.** Fluorescence and DIC image of SupraCell-Uio66-NH$_2$ based on Raw 264.7 cells for intracellular NO sensing.
Figure S3.35. Schematic illustration of NO activation pathway. Lipopolysaccharide (LPS) cross the MOF layer and bind with the toll-like receptor 4 (TLR4) on Raw 264.7 macrophage cells. Activation of TLR4 by LPS leads to the NF-κB activation and induced the expression of inducible nitric oxide synthase (iNOS), and then release NO. Released NO quenches the fluorescence of the UiO-66-NH₂ MOF NPs.
Figure S3.36. Schematic illustration of in-situ SEM electrical characterization.
Appendix 3 was adapted from

Jimin Guo‡, Wei Zhu‡*, Rita E. Serda, Jacob Ongudi Agola, Achraf Noureddine, Evelyn Ploetz, Stefan Wuttke* and C. Jeffrey Brinker* Armored red blood cells: multifunction integration through modular super-assembly of hybrid nanoparticle-based building blocks. (‡Authors contributed equally to this work)
3.1 Supplementary Figures

<table>
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<tr>
<th>pH</th>
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Figure S4.1 | Zeta potential of MOF NPs at different pH.

Figure S4.2 | Optical image of native RBCs and Armored RBC-Uio-66-NH$_2$. 


Figure S4.3 | Bright field image of Armored RBC-UiO-66-NH$_2$.

Figure S4.4 | FTIR spectra of UiO66-NH$_2$ NPs, native RBCs, and Armored RBC-UiO-66-NH$_2$. 
Figure S4.5 | Bright field image of Armored RBC-MIL-100(Fe).

Figure S4.6 | UV-Vis spectra of the oxygenated and deoxygenated states of the native RBCs.
**Figure S4.7** | The optical image of chicken embryo.

**Figure S4.8** | TEM image of the NP of MSN@ZIF-8.
Figure S4.9 | The flow of native RBCs in the capillary. Scale bars, 20 μm.

Figure S4.10 | Semilog plot of the circulation time of the MSN@ZIF-8 NPs and the related Armored RBCs.
Figure S4.11 | TEM image of the NPs of Fe₃O₄@ZIF-8.
Appendix 4 was adapted from

4.1 Supplementary Figures

**Figure S5.1** | **A**) Scanning Electron Microscopy (SEM) images of fixed RBC, silica RBC replica, silica RBC replica with polymer, and RBC polymer replica. Scale bar=2μm. **B**) Zeta potential (ζ) changes during layer by layer polymer assembly, **C**) SEM images of silica RBC replica@polymer during the silica etching process to form RBC-polymer replica. Diluted buffered oxide etch (1:10 diluted BOE) was used to etch the silica part from the silica RBC replica@polymer. Scale bar=5μm.
Figure S5.2 | Si measurement by ICP-MS of silica RBC replica@polymer during the silica etching process to form RBC-polymer replica. Diluted buffered oxide etch (1:10 diluted BOE) was used to etch the silica part from the silica RBC replica@polymer.
Figure S5.3 | A) Blood types, antigen present on the RBC, and corresponding antibodies-mediated agglutination. B) Illustration of antibodies-mediated agglutination. C) The bright field images of human type A⁺ RBCs (RBC-A⁺) and RRBC particles with human type A⁺ RBC membrane ghosts (RRBC-A⁺) incubated with different anti-typing sera for 30 minutes. Scale bar=25μm.
Figure S5.4 | The bright field images of human type B+ RBCs (RBC-B+) and RRBC particles with human type B+ RBC membrane ghosts (RRBC-B+) incubated with different anti-typing sera for 30 minutes. Scale bar=25μm.
Figure S5.5 | The fluorescence and bright field microscopy images of RBCs and RRBC particles stained with anti-ICAM-4 and anti-CD47 antibodies. Scale bar=25μm.
Figure S5.6 | A) Percentage of lysed human red blood cells after exposure (for 2h at 37°C) to 8×10^6 and 4×10^7 particles/mL of different RBC shaped particles (silica RBC replica[a], silica RBC replica@polymer[b], RBC-polymer replica[c], silica RBC replica@polymer-RBC ghost (silica-RRBC) [d], and RRBC[e]). Mean ± standard deviation, n = 3. B) HUVEC cell viability after incubated with different RBC shaped particles, C) Raw 264.7 cell viability after incubated with different RBC shaped particles.
**Figure S5.7** | Hemolysis activity of α-hemolysin alone or preincubated with RRBC particles.

**Figure S5.8** | Luminescence of RBC in different hemolysis content after incubation with biosensor loaded RRBC particles.
Table S5.1 | Simulation of flow in the microfluidic blood capillary model.[1]

<table>
<thead>
<tr>
<th>Average pressure drop across central microchannels, mbar</th>
<th>Flow rate at high pressure outlet, μL min⁻¹</th>
<th>Flow rate at low pressure outlet, μL min⁻¹</th>
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<td>2.2</td>
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3.2 Supplementary references