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Fluoro-Silane as a Functional Monomer for Protein Conformational Imprinting

Yun Peng
Utah State University

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FLUORO-SILANE AS A FUNCTIONAL MONOMER FOR PROTEIN

CONFORMATIONAL IMPRINTING

by

Yun Peng

A dissertation submitted in partial fulfillment
of the requirements for the degree
of
DOCTOR OF PHILOSOPHY
in
Biological Engineering

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Logan, Utah
2011
ABSTRACT

Fluoro-silane as a Functional Monomer for Protein Conformational Imprinting

by

Yun Peng, Doctor of Philosophy

Utah State University, 2011

By using the technology of molecularly imprinted polymer (MIP), we propose to synthesize a protein conformational imprint that also acts as a plastic enzyme, inducing protein structural transitions. The imprint aims at MIP-induced stabilization and / or formation of bound protein secondary structure and the applications associated with analysis and correction of misfolded proteins. The screening of polymeric functional monomers being able to induce the conformational transitions in proteins is investigated in this report.

The fluoro-silanes (3-heptafluoroisopropoxy)propylethoxysilane (7F) and 3,3,3-trifluoropropylmethoxysilane (3F) were employed as functional monomers for synthesis of this catalytic protein conformational imprint via sol-gel reactions. 3F was demonstrated superior to 7F for fluoro-modification of tetraethylorthosilicate (TEOS) gel in terms of retaining gel transparency and increasing hydrophobicity while maintaining a uniform distribution of encapsulated protein. Both hydrolyzed 3F and polymerized 3F exhibited strong influences on structure transitions of three template proteins: bovine
serum albumin (BSA), beta-lactoglobulin (BLG), and bovine carbonic anhydrase (BCA). The formation of molten globule intermediates that stabilized by increased alpha-helices was induced by the trifluoro-silane in BLG and BCA. Additionally, 3F was effective at a lower concentration than the benchmark fluoro-alcohol 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), validating the application of 3F as a functional monomer for protein conformational imprinting.
以此献给我的母亲及爱我的家人
ACKNOWLEDGMENTS

I would like to offer my sincerest gratitude to Dr. David W. Britt, who gave me invaluable guidance and advice through my entire graduate research period. I would also like to thank all of the members of my dissertation committee, Dr. Timothy E. Doyle, Dr. Marie K. Walsh, Dr. Soonjo Kwon, and Dr. Jixun Zhan, for their professional opinions and lab support. Special thanks are given to Dr. Kwon for his long-term support in the lab and to Dr. Walsh for her kindly help on the enzyme activity assays. Great thanks are given to Dr. Timothy A. Taylor for his constantly standing by me and willingness to help whenever needed. Also, I would like to acknowledge Russell and Hui Apple for giving me helpful comments. You are thoughtful friends.

Finally, I would like to dedicate this work to my family. To my husband Ang Shen, I thank you for always being there for me. To my lovely daughters Li and Zi, I thank you for bringing me happiness every day; you are my angels. To my sister Tao Peng and my brother Zhang hui Peng, I thank you for your understanding and encouragement every step of the way. Most importantly, to my mother, Yue ying Wang, I thank you for your endless love. This work could not have been accomplished without your unconditional and unending help through all these years.

Yun Peng
<table>
<thead>
<tr>
<th>CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT .......................................................... iii</td>
</tr>
<tr>
<td>DEDICATION .................................................................. v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS ................................................................ vi</td>
</tr>
<tr>
<td>LIST OF TABLES ................................................................ xi</td>
</tr>
<tr>
<td>LIST OF FIGURES ................................................................ xii</td>
</tr>
<tr>
<td>LIST OF SCHEMES ................................................................ xvi</td>
</tr>
<tr>
<td>ABBREVIATIONS ................................................................ xvii</td>
</tr>
<tr>
<td>CHAPTER</td>
</tr>
<tr>
<td>1. INTRODUCTION ...................................................................................... 1</td>
</tr>
<tr>
<td>PROTEIN CONFORMATIONAL IMPRINTING ...................................................... 1</td>
</tr>
<tr>
<td>HYPOTHESIS AND OBJECTIVES ................................................................. 5</td>
</tr>
<tr>
<td>DISSERTATION OUTLINE ........................................................................ 7</td>
</tr>
<tr>
<td>REFERENCES ......................................................................................... 9</td>
</tr>
<tr>
<td>2. LITERATURE REVIEW ......................................................................... 11</td>
</tr>
<tr>
<td>DEVELOPMENT OF MOLECULARLY IMPRINTED POLYMERS ......................... 11</td>
</tr>
<tr>
<td>MOLECULARLY IMPRINTED POLYMERS SYNTHESIS STRATEGIES ...................... 12</td>
</tr>
<tr>
<td>PROTEIN IMPRINTING: TOWARD ARTIFICIAL ANTIBODIES / ENZYMES ............ 15</td>
</tr>
<tr>
<td>POLYMERS FOR PROTEIN SELECTIVE MOLECULAR IMPRINTING .................... 16</td>
</tr>
<tr>
<td>Acrylamide-based MIPs ........................................................................ 16</td>
</tr>
<tr>
<td>Stimuli-sensitive hydrogel .................................................................. 19</td>
</tr>
<tr>
<td>Sol-gel ....................................................................................... 22</td>
</tr>
<tr>
<td>3-Aminophenylboronic acid (APBA) based MIPs ...................................... 25</td>
</tr>
<tr>
<td>FUNCTIONAL MONOMERS FOR NON-COVALENT MIP ................................... 27</td>
</tr>
<tr>
<td>Functional monomer-template interaction .............................................. 27</td>
</tr>
<tr>
<td>Commonly used functional monomers ................................................. 28</td>
</tr>
<tr>
<td>Hydrophobic interaction in monomer-template complex .................... 30</td>
</tr>
<tr>
<td>Optimization of pre-polymerization complex: ratios of functional monomer/cross-linker and of functional</td>
</tr>
</tbody>
</table>
Functional monomer selection strategy .......................................................... 33

PROTEIN SELECTIVE IMPRINTING APPLICATIONS ........................................ 35

Analytical separation ...................................................................................... 35
Biosensors .......................................................................................................... 38

FLUORO-ALCOHOL-PROTEIN INTERACTION:
MOTIVATION FOR APPLYING FLUORO-SILANE AS FUNCTIONAL MONOMER ............ 41

Fluoro-alcohol-induced protein structure transition ........................................ 41
Mechanisms for fluoro-alcohol-induced protein structural transitions ............. 46
From fluoro-alcohol to fluoro-silane .............................................................. 50

REFERENCES .................................................................................................... 50

3. BOVINE SERUM ALBUMIN ENCAPSULATION AND CONFORMATION IN FLUORO-MODIFIED SILICA GELS ................................................................. 68

ABSTRACT ......................................................................................................... 68
INTRODUCTION ................................................................................................. 69
EXPERIMENTAL ................................................................................................. 71

Materials ........................................................................................................... 71
Gel preparation and optimization ................................................................. 72
Fluorescence microscopy ............................................................................. 73
Fluorescence spectroscopy ....................................................................... 74
Gel surface hydrophobicity ........................................................................ 75

RESULTS AND DISCUSSION .......................................................................... 76

TEOS sol-gel synthesis ............................................................................... 76
Protein distribution in fluoro-silane/TEOS gel ..................................... 80
Surface hydrophobicity of fluoro-silane/TEOS gel ................................ 81
Hydrolyzed fluoro-silane interaction with BCA ..................................... 82
Fluoro-silane interaction with BSA in gel ........................................ 83
Structure-inducing activities of 3F and 7F .............................................. 85

CONCLUSIONS ................................................................................................. 88
REFERENCES .................................................................................................... 89

4. TRIFLUOROSILANE INDUCES STRUCTURAL CHANGES IN BETA-LACTOGLOBULIN: FUNCTIONAL MONOMER FOR CATALYTIC PROTEIN CONFORMATIONAL IMPRINTING ......................................................................................... 94
ABSTRACT ........................................................................................................... 94
INTRODUCTION ................................................................................................. 95
EXPERIMENTAL .................................................................................................. 98

Materials .................................................................................................................. 98
Sample preparation .................................................................................................. 98
Trp and 1,8-ANS fluorescence in pre-polymerization complex ................................ 99
1,8-ANS fluorescence in BLG-entrapped TEOS gel ............................................. 100
Circular dichroism spectroscopy ........................................................................... 101

RESULTS AND DISCUSSION ............................................................................. 102

3F-induced structural transitions in BLG ............................................................... 102
Conformation induction in fluoro- and alkyl-sol-gels ......................................... 108
Modes of structure inducing activity .................................................................... 111

CONCLUSIONS ................................................................................................... 112
REFERENCES ........................................................................................................ 113

5. ENZYME ACTIVITY AND CONFORMATIONAL CHANGES OF BOVINE CARBONIC ANHYDRASE IN TRIFLUOROSILANE MODIFIED SOL-GEL ........................................................................................................... 117

ABSTRACT ........................................................................................................... 117
INTRODUCTION ................................................................................................. 118
EXPERIMENTAL .................................................................................................. 120

Materials and sample preparation ........................................................................ 120
BCA enzyme activity in solution .......................................................................... 121
BCA activity in TEOS gels with urea treatments .................................................. 121
BCA activity in 3F-modified TEOS gel ................................................................. 123
Fluorescence spectroscopy .................................................................................... 123

RESULTS AND DISCUSSION ............................................................................. 125

BCA denaturation and renaturation in TEOS gel ................................................... 125
Hydrolyzed 3F-BCA interaction .......................................................................... 127
Polymerized 3F-BCA interaction ........................................................................ 134
Enzyme activity in 3F-modified gel ...................................................................... 136

CONCLUSIONS ................................................................................................... 139
REFERENCES ........................................................................................................ 140

6. CONCLUSIONS AND FUTURE WORK ........................................................... 144

CONCLUSIONS ................................................................................................... 144
3F-PROTEIN INTERACTION MODEL ................................................................... 145
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 1</td>
<td>The chemical structures of fluoro-alcohols (HFIP and TFE), silanes and fluoro-silanes that applied as the cross linker or the functional monomers for synthesis of protein conformational imprinting via sol-gel reactions</td>
</tr>
<tr>
<td>2.1</td>
<td>Characteristics of covalent imprinting and non-covalent imprinting</td>
</tr>
<tr>
<td>2.2</td>
<td>TFE- and HFIP-induced structure effects on peptides and proteins</td>
</tr>
<tr>
<td>2.3</td>
<td>Chemical properties of water and various alcohols</td>
</tr>
<tr>
<td>3.1</td>
<td>Sessile water contact angles ($\theta_{\text{H}_2\text{O}}$) on surface of TEOS gels containing 4.5 $\mu$M BSA and modified with 7F or 3F at 5% and 10% (fluoro-silane/TEOS, mol%)</td>
</tr>
<tr>
<td>6.1</td>
<td>Approaches for protein-selective surface imprinting</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>2.1</td>
<td>Schematic representation of template-induced shrinking of the stimuli-sensitive hydrogel during the protein rebinding with its MIP</td>
</tr>
<tr>
<td>2.2</td>
<td>Common functional monomers used in non-covalent molecular imprinting</td>
</tr>
<tr>
<td>3.1</td>
<td>Gelation time at neutral and slightly basic pH values as a function of the ratio of hydrolyzed TEOS sol to 0.01M hepes condensation buffer</td>
</tr>
<tr>
<td>3.2</td>
<td>Dependence of gel turbidity on the ratio of hydrolyzed TEOS sol to condensation buffer for silica gels prepared by 0.01M pH 7 and pH 8 hepes buffer</td>
</tr>
<tr>
<td>3.3</td>
<td>The turbidity of TEOS silica gel doped with either 5% or 10% (M/M) 3F/7F</td>
</tr>
<tr>
<td>3.4</td>
<td>Fluorescence microscopy reveals the strong influence of the volume ratio of hydrolyzed silane to 0.01M pH 8 hepes condensation buffer on the distribution of 4.5 μM FITC-BSA in pure TEOS gel and 10% 3F or 10% 7F modified TEOS gels</td>
</tr>
<tr>
<td>3.5</td>
<td>The fluorescence intensity of protein Trp residues and the probe 1,8-ANS as a function of the concentration of hydrolyzed silane (TEOS, 3F and 7F) or fluoro-alcohol HFIP and TFE</td>
</tr>
<tr>
<td>3.6</td>
<td>Increased fluorescence of 90 μM 1,8-ANS after a two-hour diffusion into 3F or 7F modified silica gels containing 4.5 μM BSA</td>
</tr>
<tr>
<td>4.1</td>
<td>The dependence of the intrinsic Trp fluorescence intensity of BLG on concentration of the fluoro-alcohols or the hydrolyzed silanes as indicated in 20 mM HCl</td>
</tr>
<tr>
<td>4.2</td>
<td>The fluorescence intensity of 0.03 mg/ml 1,8-ANS as the function of the concentration of fluoro-alcohols (HFIP, TFE) or hydrolyzed silanes (3F, nPM and TEOS) in presence of 0.37 mg/ml BLG in 20 mM HCl</td>
</tr>
<tr>
<td>4.3</td>
<td>The energy transfer efficiency between 1,8-ANS and BLG Trp residues, presented as the ratio of 1,8-ANS fluorescence to the intrinsic fluorescence of ANS-bound BLG (I_{ANS}/I_{Trp-ANS})</td>
</tr>
<tr>
<td>4.4</td>
<td>The structure of bovine beta-lactoglobulin (BLG)</td>
</tr>
</tbody>
</table>
4.5 Circular dichroism spectrum of 3F-induced alpha-helical structure (minima at 208 and 222 nm) over the silane concentration range as indicated.................................................................108

4.6 The fluorescence intensity of 1,8-ANS binding with BLG in TEOS gel either entrapped with the fluoro-alcohols (HFIP and TFE) or copolymerized with 3F or nPM ..................................................................................109

4.7 The chemical structures of the silanes (TEOS, nPM and TEOS) and silanols hydrolyzed from each silane by acid catalysis ..............................................112

5.1 The activities of 0.025 mg/ml BCA in 0.01 M pH 7.5 hepes buffer or in TEOS silica gel (pH 7.5) with different urea treatments ......................................126

5.2 The enzyme activities of 0.025 mg/ml BCA in TEOS gel balanced with 0.01 M pH 7.5 hepes buffer as a function of the doping ratio of 3F or nPM in TEOS gel ........................................................................128

5.3 The changes of the fluorescence intensity of 0.03 mg/ml 1,8-ANS as a function of the concentration of hydrolyzed 3F or nPM in 0.01 M pH 2.5 hepes buffer at indicated time of interaction ........................................130

5.4 The changes of the fluorescence intensity of 0.03 mg/ml 1,8-ANS as a function of concentration of hydrolyzed 3F or nPM in 0.01 M pH 4.5 hepes buffer at indicated time of interaction ........................................132

5.5 The fluorescence intensity of 0.03 mg/ml 1,8-ANS as a function of the concentration of HFIP or TFE in 0.01 M pH 2.5 hepes buffer and at interaction time intervals as indicated ...................................................135

5.6 The fluorescence intensity of 1,8-ANS after diffusion in 200 μl TEOS gel either modified with 3F or nPM or entrapped with HFIP and TFE ...............137

5.7 The structure and the enzyme active site cavity of bovine carbonic anhydrase obtained from x-ray crystallography ..............................................138

6.1 Schematic representation for CRP micro-contact imprinting and rebinding .................................................................................................153

6.2 Schematic representation for synthesis of the protein-imprinted siloxane polymer on chitosan microsphere covalently immobilized with protein template ........................................................................154

6.3 Surface imprinting of protein on magnetic particles by covalent immobilization of template protein .........................................................155

6.4 Schematic representation of the nano-rods protein imprinting on gold electrode ..................................................................................156
6.5 The protocol for epitope imprinting of proteins on glass or oxidized silicon surfaces

A.1 The gelation time as the function of the pH of 0.01M hepes buffer used for preparation of TEOS silica gel and mixed with hydrolyzed TEOS sol at the indicated volume ratios (sol / buffer)

A.2 The changes of 1,8-ANS fluorescence with the time of diffusing in 3F or 7F modified silica gels.

A.3 The Trp fluorescence of BSA and the fluorescence of 1,8-ANS binding with BSA in presence of HFIP and TFE in 0.01 M pH 2.5 hepes buffer

B.1 The fluorescence intensity of 1,8-ANS binding with BLG in TEOS gel either entrapped with the fluoro-alcohols (HFIP and TFE) or copolymerized with 3F or nPM.

B.2 The dependence of fluorescence intensity of BLG Trp residues on the concentration of fluoro-alcohols (HFIP / TFE) entrapped in TEOS gel.

B.3 The dependence of the fluorescence intensity of 1,8-ANS on the concentration of fluoro-alcohols (HFIP / TFE) entrapped in TEOS as the probe diffused in the gels at indicated time intervals.

B.4 The dependence of the fluorescence intensity of 1,8-ANS on doping concentration of 3F or nPM as the probe diffused in the gels at indicated time intervals.

B.5 The fluorescence spectrum of 1,8-ANS in TEOS gel doped with various concentration of 3F.

B.6 The fluorescence spectrum of 1,8-ANS in TEOS gel doped with various concentration of nPM.

C.1 The effects of hydrolyzed 3F or hydrolyzed nPM on fluorescence of BCA Trp residues after interacting with the protein in 0.01 M pH 4.5 hepes buffer.

C.2 The dependence of the fluorescence intensity of 20 μM BCA Trp residues on concentration of various cosolvents (HFIP, TFE, hydrolyzed 3F, hydrolyzed nPM) in 0.01 M pH 2.5 hepes buffer.

C.3 The dependence of the fluorescence intensity of 1,8-ANS on concentration of various cosolvents (HFIP, TFE, hydrolyzed 3F, hydrolyzed nPM) in 0.01 M pH 2.5 hepes buffer.

C.4 The fluorescence intensity of BCA Trp residues in TEOS gel either
entrapped with the fluoro-alcohols (HFIP and TFE) or copolymerized with 3F or nPM

C.5 The dependence of the fluorescence intensity of 1,8-ANS on the concentration of fluoro-alcohols (HFIP / TFE) entrapped in TEOS gel as the probe diffused in the gels at indicated time intervals

C.6 The dependence of fluorescence intensity of BCA Trp residues on the concentration of fluoro-alcohols (HFIP / TFE) entrapped in TEOS gel

C.7 The pH dependence of 1,8-ANS fluorescence binding with BCA in 0.01 M hepes buffer

C.8 The fluorescence spectrum of 1,8-ANS binding with BCA in 0.01 pH 3 hepes buffer

C.9 The fluorescence spectrum of 1,8-ANS in 3F-doped TEOS gel

C.10 The fluorescence spectrum of 1,8-ANS in nPM-doped TEOS gel
# LIST OF SCHEMES

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 The synthesis of molecularly imprinted polymers (MIPs)</td>
<td>2</td>
</tr>
<tr>
<td>1.2 The fabrication of catalytic protein conformational imprint on the surface of support beads coated with fluoro-silane modified sol-gel</td>
<td>4</td>
</tr>
<tr>
<td>6.1 Model showing 3F-protein interactions and 3F-induced protein unfolding and formation of alpha-helix based on a two-state transition mechanism</td>
<td>147</td>
</tr>
<tr>
<td>6.2 Schematic presentation of thermal response hydrogel imprinting coated on electromagnetic nanoparticle</td>
<td>159</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>AA</td>
<td>acrylic acid</td>
</tr>
<tr>
<td>AAm</td>
<td>acrylamide</td>
</tr>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>Am</td>
<td>acrylamide</td>
</tr>
<tr>
<td>AMPSA</td>
<td>2-acrylamido-2-methyl-1-propanesulfonic acid</td>
</tr>
<tr>
<td>APBA</td>
<td>3-aminophenylboronic acid</td>
</tr>
<tr>
<td>APS</td>
<td>γ-aminopropyltriethoxysilane</td>
</tr>
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<td>APTMS</td>
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<tr>
<td>BCA</td>
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<td>Beta-CD</td>
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</tr>
<tr>
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<td>beta-lactoglobulin</td>
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<tr>
<td>BLV</td>
<td>bovine leukemia virus</td>
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<tr>
<td>BODIPY</td>
<td>arylazide-derivatized luminophore</td>
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<td>BSA</td>
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</tr>
<tr>
<td>CEC</td>
<td>capillary electro-chromatography</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CS</td>
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</tr>
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<td>Cyt c</td>
<td>cytochrome c</td>
</tr>
<tr>
<td>C8-TMOS</td>
<td>n-octyltrimethoxysilane</td>
</tr>
<tr>
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<td>2,4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>DSC</td>
<td>differential scanning calorimetry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GO</td>
<td>glucose oxidase</td>
</tr>
<tr>
<td>EGDMA</td>
<td>ethylene glycol dimethacrylate</td>
</tr>
<tr>
<td>3F</td>
<td>(3,3,3-trifluoropropyl)trimethoxysilane</td>
</tr>
<tr>
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<td>(3-heptafluoroisopropoxy)propaethoxysilane</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform inferred spectroscopy</td>
</tr>
<tr>
<td>HAPTS</td>
<td>bis(2-hydroxyethyl)-aminopropytriethoxysilane</td>
</tr>
<tr>
<td>HEMA</td>
<td>2-hydroxyethyl methacrylate</td>
</tr>
<tr>
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</tr>
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</tr>
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<td>HgH</td>
<td>human growth hormone</td>
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<td>interleukin-1 beta</td>
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<td>L-phenylalanine anilide</td>
</tr>
<tr>
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<td>lysozyme</td>
</tr>
<tr>
<td>MAA</td>
<td>methacrylic acid</td>
</tr>
<tr>
<td>MAH</td>
<td>N-methacryloyl-(L)-histidinemethylester</td>
</tr>
<tr>
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<td>myoglobin</td>
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<tr>
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<td>N,N-methylenbisacrylamide</td>
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<td>mesoporous chloromethylated polystyrene</td>
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<tr>
<td>MIP</td>
<td>molecularly imprinted polymer</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
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</tr>
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<td>MISPE</td>
<td>Molecular imprinted solid phase extraction</td>
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<td>Methyl methacrylate</td>
</tr>
<tr>
<td>NEP</td>
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</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
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<td>Non-imprinted polymer</td>
</tr>
<tr>
<td>NIPAm</td>
<td>N-isopropylacrylamide</td>
</tr>
<tr>
<td>NOE</td>
<td>Nuclear Overhauser effect spectroscopy</td>
</tr>
<tr>
<td>nPM</td>
<td>N-propyltrimethoxysilane</td>
</tr>
<tr>
<td>4NPPC</td>
<td>O-4-nitrophenylphosphorylcholine</td>
</tr>
<tr>
<td>PAA</td>
<td>Polyacrylamide</td>
</tr>
<tr>
<td>PAD</td>
<td>Pulsed amperometric detection</td>
</tr>
<tr>
<td>PCM</td>
<td>Polycarbonate membrane</td>
</tr>
<tr>
<td>PEDOT</td>
<td>Poly (3, 4-ethylenedioxythiophene)</td>
</tr>
<tr>
<td>PHEMAT</td>
<td>Hydroxyethyl methacrylate-N-methacryloyl-L-tyrosine methyl ester</td>
</tr>
<tr>
<td>PIXIES</td>
<td>Protein imprinted xerogels with integrated emission sites</td>
</tr>
<tr>
<td>PQC</td>
<td>Piezoelectric quartz crystal</td>
</tr>
<tr>
<td>PQCI</td>
<td>Piezoelectric quartz crystal impedance</td>
</tr>
<tr>
<td>PSS</td>
<td>Polystyrene sulphonate</td>
</tr>
<tr>
<td>PTMS</td>
<td>Propyltrimethoxysilane</td>
</tr>
<tr>
<td>QCM</td>
<td>Quartz crystal micro-gravimetry</td>
</tr>
<tr>
<td>RFGD</td>
<td>Radio frequency glow-discharge</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SIP</td>
<td>Surface initiated radical polymerization</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SMO</td>
<td>sulfamethoxazole</td>
</tr>
<tr>
<td>SMZ</td>
<td>sulfamethazine</td>
</tr>
<tr>
<td>SpA</td>
<td>Staphylococcus aureus protein A</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N-tetramethylene diamine</td>
</tr>
<tr>
<td>TEOS</td>
<td>tetraethylorthosilicate</td>
</tr>
<tr>
<td>Tf</td>
<td>transferrin</td>
</tr>
<tr>
<td>TFE</td>
<td>2,2,2-trifluoroethanol</td>
</tr>
<tr>
<td>TFMAA</td>
<td>(2-trifluoromethyl)acrylic acid</td>
</tr>
<tr>
<td>TMOS</td>
<td>tetramethylyorthosilicate</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>4-VP</td>
<td>4-vinylpyridine</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

PROTEIN CONFORMATIONAL IMPRINTING

The proper folding of protein structures is of vital importance to their biological functions. The well-defined three-dimensional conformation of protein is coupled essentially with many biological processes, ranging from molecular recognition and transportation to cell growth and regulation. Misfolded proteins link to a wide variety of pathological conditions, including some of the most perplexing medical problems such as Alzheimer’s disease, Parkinson’s disease and the prion encephalopathies. In spite of the diverse clinical presentations, the molecular basis of the neurodegenerative disorders usually involves destabilization of alpha-helices and formation of beta-sheets, which consequently lead to the multimerization of misfolded proteins into insoluble, extracellular aggregates and / or intracellular inclusions such as cross beta-sheet amyloid fibrils. Thus, stabilizing the alpha-helix and / or disrupting formation of beta-sheet and the subsequent self-association of different pathological macrostructures are important therapeutic strategies for protein conformational diseases.

Molecular imprinting is the synthesis of a highly cross-linked polymer containing template molecule-induced binding cavities that are complementary to both spatial configuration and functionality of the template molecule(s) (Scheme 1.1). Such a molecularly imprinted polymer (MIPs) keeps a memory of the template molecule and can be “programmed” to recognize various target molecules with biological receptor-mimic affinity and selectivity. Since MIP technology was developed half a century ago, it has been successfully used in preparing materials with high selectivity for a wide range of
low molecular weight compounds such as steroids, monosaccharides, amino acids and their derivatives. MIPs applications in the fields of drug discovery, drug delivery, analytical separation, biosensing and synthetic antibodies / receptors also have been widely reported.9-12 Due to the large size and the structure complexity of protein molecules, the development of protein-selective imprinting is much slower than that of small molecule imprinting. However, protein-imprinted MIPs have become attractive research topics for both academic and industry communities due to continuously growing interest and demand for exploiting natural recognition mimic systems for biomedical applications.

Here we propose to apply MIP technology to synthesize a protein conformational imprint that specifically binds with target protein conformation and, more importantly, acts as a plastic enzyme to stabilize the protein conformation to non-pathogenic

**Scheme 1.1** The synthesis of molecularly imprinted polymers (MIPs).
alpha-helices. Such a “plastic enzyme” may be applied as an alternative recognition element for analysis and correction of misfolded proteins (Scheme 1.2). In this aspect, the selection of functional monomers is primarily important, as the recognition specificity, selectivity and the function of protein-imprinted polymers are affected to a large extent by the properties and the three-dimensional distribution of functional monomers and their interaction with template proteins. Thus, a functional monomer must be screened first for protein affinity and for conformation-inducing activity in bulk solution, then it can be demonstrated effective when polymerized in MIP.

The fluorinated alcohols 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and 2,2,2-trifluoroethanol (TFE) have long been recognized as having prominent effects on interrupting native protein structures by weakening hydrophobic interactions and minimizing the exposure of peptide backbone, which consequently strengthens the helical propensities. In this study, (3-heptafluoroisopropoxy)propylethoxysilane (7F) and 3,3,3-trifluoropropylmethoxysilane (3F), the structural analogues of HFIP and TFE, respectively (Table 1.1), were employed as functional monomers to bind with the target proteins and guide their structural transitions toward stabilized alpha-helices. The spatial arrangement of functional monomers around the template protein was then locked by polymerizing in a tetraethylorthosilicate (TEOS) matrix. The template protein can be released by crushing the sol-gel and exposing the binding sites. Alternatively, a surface imprinting approach can be employed as indicated in Scheme 1.2. This research focused on investigating the efficiency of selected fluoro-silanes as polymerizable analogues of fluoro-alcohols to synthesize the catalytic protein conformational imprint. Three proteins (beta-lactoglobulin, bovine serum albumin and bovine carbonic anhydrase) that have
have been widely used for studies on protein folding and unfolding were employed as the model proteins. The structure-inducing activity efficiencies of the fluoro-silanes were demonstrated by fluorescence spectroscopic assays on extrinsic and intrinsic fluorophores that are sensitive to protein conformation. HFIP and TFE were employed as the benchmark controls. The interaction of a template protein with a hydrolyzed fluoro-silane in pre-polymerization complex was characterized in acidic condition. TEOS cross-linkers

**Scheme 1.2** The fabrication of catalytic protein conformational imprint on the surface of support beads coated with fluoro-silane modified sol-gel. The template protein first interacts with the fluoro-silane functional monomers to form a pre-polymerization complex, which is subsequently polymerized with the sol-gel derived silica gel on the surface of the support beads. After elution of the template protein, imprinted cavities form on the gel surface to bind specifically with the target protein and act as plastic enzymes, inducing the structure transition from beta-sheets to alpha-helices.
Table 1.1 The chemical structures of fluoro-alcohols (HFIP and TFE), silanes and fluoro-silanes that applied as the cross linker or the functional monomers for synthesis of protein conformational imprinting via sol-gel reactions.

(a) 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)  
(b) 2,2,2-trifluoroethanol (TFE)  
(c) Tetraethylorthosilicate (TEOS)  

(d) (3-heptafluoroisopropoxy) propylethoxysilane (7F)  
(e) 3,3,3-trifluoropropyl methoxysilane (3F)  
(f) n-propyltrimethoxy silane (nPM)

were then added to freeze spatial arrangement of the fluoro-silane around the template, and their interactions in the gels were investigated. Molecular modeling was used to illustrate possible modes of fluoro-silane-protein interaction where “binding clefts” along the alpha-helices favorably accommodate and interact with fluoro, alkyl and silanol groups of 3F. 7F was less effective due to its higher hydrophobicity that favors the silane self-aggregation and the possible steric exclusion from the purported protein binding sites.

**HYPOTHESIS AND OBJECTIVES**

This research is based on the hypothesis that fluoro-silanes 3F and 7F behave as the respective fluoro-alcohol analogues TFE and HFIP by unfolding proteins and
inducing the formation and/or stabilization of alpha-helices in the target proteins. The main objective of this research is to demonstrate the structure-inducing activity of selected fluoro-silanes and to validate the applicability of the fluoro-silanes as functional monomers for synthesis of the protein conformational imprint.

To verify the hypothesis, a series of experiments have been designed to characterize the solution behavior of hydrolyzed fluoro-silanes and to optimize their incorporation into TEOS silica gels. The experimental work of this research has been divided into three specific aims:

1) *Optimization of conditions for synthesis of fluoro-silane modified TEOS gel.* The sol-gel reaction pH and the ratio of the buffer to silane sol were varied to construct a fluoro-silane modified TEOS gel with rapid gelation rate, optical transparency and uniform distribution of the template protein.

2) *Interaction of template proteins with hydrolyzed fluoro-silane monomers.* The solution pH was selected where fluoro-silanes underwent rapid hydrolysis but slow condensation, allowing sufficient time for monomer-protein interaction. HFIP and TFE were used as benchmark controls to assess the efficiency of structure-inducing activities of the fluoro-silanes. The influences of fluoro, alkyl and silanol groups on protein structures were selectively probed by using the fluoro-alcohols, the propyl substitute of TEOS (n-propyltrimethoxysilane, nPM) and pure TEOS as the controls (Table 1.1).

3) *Preservation of fluoro-silane-protein complex and the structure-inducing activity by polymerization in a TEOS matrix.* Multiple fluoro-silanes likely bind to defined regions of a given protein. This arrangement needs to be preserved in the molecularly imprinted pockets to form a catalytic binding site. The construction of catalytic binding pockets can
be inferred through distinct conformational transitions in the template protein(s). This also reflects the association of retained activity of the polymer-anchored fluoro-alkyl groups and the porous structure of the silica gel.

**DISSECTATION OUTLINE**

In chapter 2, a review of the literature on development of MIP technology and on imprinting of macromolecules is presented. An overview is given for two typical strategies of MIP technology based on covalent or non-covalent interactions between templates and functional monomers. Given the aims of this thesis, protein-selective MIPs are reviewed elaborately in terms of the polymers and the interactions of the functional monomers with proteins. Sol-gel technology and hydrophobic functional monomers used in protein-based MIP are also presented. After this, the major applications of protein-selective MIPs for analytical separation and biosensor fabrication are reviewed. The last part of the chapter provides a review on the interactions of proteins with the fluoro-alcohols HFIP or TFE, which is necessary to understand protein conformational imprinting. The research is presented as three papers / chapters to address aim one through three.

Chapter 3 presents the results for condition optimization of the synthesis of fluoro-silane modified TEOS gel in terms of buffer pH and the ratio of buffer to hydrolyzed silane sol (buffer : sol). Buffer pH, ratios of fluoro-silane to TEOS and silane concentration were systematically varied to form optically transparent gels with sufficient mechanical properties. Fluorescein isothiocyanate-conjugated bovine serum albumin (FITC-BSA) was used as the template in gel optimization studies so that fluorescence microscopy could be used to determine the conditions that result in the uniform
distribution of protein in the gel. The interaction of BSA with 3F or 7F were monitored by fluorescence spectroscopic assays on protein tryptophan (Trp) residues and the probe 1,8-ANS, which has high affinity with the hydrophobic pockets of protein and has been used widely for detecting molten globule intermediate. In the results of this chapter, 3F showed superior properties to 7F for modification of TEOS gel in terms of the optically transparency and the uniform protein distribution. Additionally, hydrolyzed 3F and polymerized 3F both led to the loss of 1,8-ANS binding sites, while 7F exhibited complex interaction with FITC-BSA, which may be associated with 7F self-aggregation due to its stronger hydrophobicity.

Chapter 4 discusses how beta-lactoglobulin (BLG) was applied as the template protein to interact with 3F. To test aim two, the interaction of BLG with hydrolyzed 3F under acidic conditions is evaluated in this chapter. The fluorescence intensity of protein Trp and 1,8-ANS, as well as the non-radiative fluorescence energy transfer between the two fluorophores, were used to demonstrate 3F-induced conformational transitions of BLG. In addition to HFIP and TFE, nPM was also applied to investigate the composition contributions of 3F to its structure-inducing activity. In support of aim two, hydrolyzed 3F induced the formation of a molten globule-like (MG-like) BLG intermediate, which circular dichroism (CD) conformed to be largely stabilized by increased content of the alpha-helices. The order of the structure-inducing activities of the fluoro-alcohols / silanes is 3F > HFIP > nPM >> TFE > TEOS. Moreover, the activity of 3F was retained upon the incorporation of the trifluoro-silane monomer in TEOS gel, which verified the third aim of this research.

In Chapter 5, the interactions of bovine carbonic anhydrase (BCA) with
hydrolyzed 3F and copolymerized 3F are assessed to further evaluate 3F-induced structural transitions. In addition to the fluorescence spectroscopy assays on 3F-induced emission intensity changes of 1,8-ANS, the influences of 3F on BCA enzyme activities in TEOS gel were also studied to demonstrate the protein structure changes. The results of this chapter are consistent with the results in Chapter 4, further verifying that either hydrolyzed 3F in pre-polymerization complex or polymerized 3F in TEOS gel induced the conformational transitions of BCA. Hydrolyzed 3F induced the formation of a stable molten globule-like (MG-like) intermediate at pH 4, while multiple MG-like intermediates were induced at pH 2.5. Moreover, polymerized 3F retained and even enhanced the structure-inducing activity of hydrolyzed 3F by being effective at a lower concentration and within a narrower concentration window, validating the application of the trifluoro-silane as the functional monomer for catalytic protein conformational imprinting.

Chapter 6 gives the conclusion for the report. A molecular model for interpretation of 3F-protein interactions is proposed. Further directions of this research are also discussed, and surface imprinting approaches associated with the future work are reviewed.

REFERENCES


CHAPTER 2
LITERATURE REVIEW

DEVELOPMENT OF MOLECULARLY IMPRINTED POLYMERS

The earliest attempts to synthesize polymers for molecular recognition were inspired by the theory of Pauling, which was formulated in the 1940s and was based on the complex question of antibody formation in the immune system.\textsuperscript{1} Pauling proposed that an antigen guided the amino acid chains of an antibody to form a shape around it via a mechanism he termed as “molecular complementarity.” Thus, the antibody would memorize a specific moiety of the antigen.\textsuperscript{2} While incorrect, this idea that a moving polymer chain could form a complementary mold around a structure inspired the field of molecularly imprinted polymers, or MIPs.

The first MIP is attributed to Dickey’s work in 1949, who polymerized dye molecules in a sodium silicate matrix.\textsuperscript{1} The dye-imprinted silica demonstrated a pronounced selectivity for the template dye versus challenger dyes. In this case, no dye-specific functional monomers were employed. In 1972, a major advancement in molecular imprinting was achieved when template “specific” functional monomers were employed in synthetic organic polymers.\textsuperscript{2,3} Wulff proposed in his work that in addition to size and spatial complementarities, functional monomers were responsible for molecular recognition by forming reversible covalent bonds with the template. Templates imprinted using this approach include glyceric acid, derivatives of mannose, galactose, fructose, sialic acid, castasterone, nucleotides, and so on.\textsuperscript{2,4-8}

Following the development of covalent MIPs in the 1980s, non-covalent MIPs
employing forces such as hydrogen bonds and electrostatic interactions have been extensively investigated.\textsuperscript{9,10} A variety of low molecular weight templates with high solubility in organic solvents have been imprinted in a range of polymers, resulting in a tremendous increase in the number of publications on MIPs in the last decade.\textsuperscript{11} A multitude of imaginative approaches to synthesize new imprint polymers have also been reported.\textsuperscript{12,13} Many of the imprinted polymers have been successively applied to electrophoresis and chromatographic analysis, solid phase extraction, enantiomers separation, drug discovery, controlled drug-release, and sensor fabrication.\textsuperscript{14-24} Molecular imprinting, after development for half a century, has therefore emerged as a unique technology for synthesis of polymeric receptors or artificial antibodies (“plastibodies”), offering a generic, robust and cost-effective alternative to existing techniques such as monoclonal antibodies.

**MOLECULARLY IMPRINTED POLYMERS SYNTHESIS STRATEGIES**

The basic principle for MIP synthesis is relatively straightforward, and most published protocols follow the same general procedure. Functional monomers are selected to be complementary to the template molecule and are mixed to form a pre-polymerization complex, which is subsequently polymerized with cross-linker monomers in the presence of a proper solvent, most often an aprotic and non-polar solvent. The mixture is cured to generate a porous, highly cross-linked and rigid material. The template molecules are then removed by washing with a solvent or a combination of chemical or enzymatic treatments, resulting in an imprinted matrix with molecular-scale cavities that are complementary in shape and functionality to the template molecules.
Molecularly imprinted polymers possess a “permanent memory” of the templates, most often demonstrated by enhanced rebinding of the template molecules to the MIPs versus non-imprinted polymer equivalent, or “NIPs.” The basic principles of molecular imprinting are illustrated in Scheme 1.1.

There are essentially two types of molecular imprinting strategies based on covalent and non-covalent interactions during the formation of pre-polymerization complex. For covalent MIPs, functional monomers are covalently bonded with template molecules to form a template-monomer complex (pre-polymerization complex). The chemical bonds ensure the correct spatial orientation of the functional groups during cross-linking. These bonds are broken during template removal, leaving the functional groups correctly positioned in the binding cavities. The first successful covalent MIPs employed a simple sugar conjugated to a boronic acid derivative. The conjugated complex was cross-linked with ethylene dimethacrylate. When the ester was cleaved, the rebinding of the template was found to be very selective. Due to the chemical linkages formed for the template-monomer complex, the binding sites tended to be homogeneous and the formation of non-template mediated binding sites in the polymer were greatly decreased. However, the relatively specific structural requirements of covalent methods limit the variety of the templates that can be imprinted. Also, the relatively harsh conditions for cleavage of the covalently bound template carry the risk of disrupting polymer rigidity.

Non-covalent MIPs were introduced a decade later than covalent MIPs and are used more extensively due to the ease of forming the template-monomer complex and extracting the template. In this approach, functional monomers and templates are mixed
and allowed to form pre-polymerization complex by “self-assembling.” Typical template-monomer non-covalent interactions include hydrogen bonding, electrostatic interactions, π–π complexation, hydrophobic interactions and metal-ligand complexation. In contrast to the harsh conditions for template removal in covalent imprinting, non-covalently imprinted template can be removed via simple diffusion (for small molecules only) usually in a polar or acidic solvent that sufficiently overcomes the non-covalent interactions between the template and the polymer. For these reasons, non-covalent imprinting allows rapid rebinding kinetics and introduction of a greater variety of functionalities into MIP binding sites. Thus, the majority of imprinting methods conducted recently, particularly the imprinting of macromolecules, involves non-covalent techniques. A summary of the characteristics of covalent and non-covalent imprinting is shown in Table 2.1.

**Table 2.1** Characteristics of covalent and non-covalent imprinting.

<table>
<thead>
<tr>
<th></th>
<th>Covalent imprinting</th>
<th>Non-covalent imprinting</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Advantages</strong></td>
<td>Low non-specific rebinding</td>
<td>Rapid and reversible rebinding</td>
</tr>
<tr>
<td></td>
<td>Homogenous binding sites</td>
<td>Template easy to remove</td>
</tr>
<tr>
<td></td>
<td>Strong and specific rebinding</td>
<td>Wide variety of functional monomers</td>
</tr>
<tr>
<td><strong>Disadvantages</strong></td>
<td>Template hard to remove</td>
<td>High non-specific binding</td>
</tr>
<tr>
<td></td>
<td>Slow rebinding kinetics</td>
<td>Heterogeneous binding sites</td>
</tr>
<tr>
<td></td>
<td>Limited suitable template varieties</td>
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</table>
Over the last 20 years, efforts to replicate macromolecular recognition behavior with synthetic polymers have received a great deal of attention due to the success observed for many low molecular weight molecules.\textsuperscript{25-27} Chemically synthesized imprints for a range of biological macromolecules and even microorganisms in aqueous solution have been explored. For instance, Mosbach’s group described the imprinting of a dipeptide and endogenous neuropeptide (Leu-enkephalin) in acrylate-based polymers.\textsuperscript{28,29} Rachkov and Minourfa fabricated an artificial hydrogel receptor for a peptide hormone, oxytocin, via the epitope imprinting method.\textsuperscript{30} Kofinas and co-workers have prepared imprints for detection of a Tobacco mosaic virus in hydrogel cross-linked by poly(allylamine hydrochloride).\textsuperscript{31} More recently, \textit{Escherichia coli}-imprinted gel granules synthesized by bulk imprinting were able to distinguish between different types and strains of bacteria by using free-zone electrophoresis.\textsuperscript{32} Dickert et al. coated the surface of quartz crystal microbalances with sol-gel for specific recognition of yeast cells.\textsuperscript{33} The modified sensor showed high sensitivity for detection of yeast and even the cell fragments in fluid conditions. Not long after this, they fabricated a imprint for erythrocyte recognition using a similar surface imprinting technique.\textsuperscript{34}

Among many macromolecules, the imprinting of protein has received the most attention owing to the growing demand for production of cost-effective, robust and reproducible protein recognition elements.\textsuperscript{35-38} Protein-selective MIPs are viewed as the alternatives to traditional antibodies and receptors, which are often expensive to produce and are subject to denaturation and aggregation over time and / or in more extreme environments. To date, the standard “model proteins” for protein-selective MIPs such as
BSA, human serum albumin (HSA), lysozyme (Lyz) and cytochrome c (Cyt c) have been intensively studied as templates.\textsuperscript{39-41} However, the preparation of protein-selective MIPs in aqueous solvents still faces a number of complications that are largely absent when targeting low molecular weight molecules. First, most well-established imprinting methods for small molecules cannot directly apply to the protein imprinting due to their poor solubility in aprotic solvents. Second, application of polar solvents weakens non-covalent bonds (e.g., electrostatic and hydrogen bonds) that link template proteins and functional monomers. Third, reduced and restricted diffusion of protein molecules complicates template extraction and rebinding. In addition, flexible protein structure is prone to change with microenvironment conditions (e.g., temperature, pH, ionic strength), making the imprinting of protein really difficult.\textsuperscript{42} Novel methods for synthesis of MIPs addressing protein size and conformational flexibility are needed.

**POLYMERS FOR PROTEIN SELECTIVE MOLECULAR IMPRINTING**

Due to the high molecule weight of proteins, as well as their surface structure complexity and the susceptibility to denaturation or aggregation, the number of polymer systems suitable for protein-selective MIPs is rather limited. Aqueous condition, mild pH and moderate temperature are primary requirements. Secondary considerations include polymer porosity, integrity and ease of protein-template removal.

**Acrylamide-based MIPs**

Polyacrylamide gels were originally used in gel-electrophoresis for separation and purification of large molecular weight proteins. For protein-selective MIPs, a hydrogen bond is often applied as a molecular recognition interaction. Owing to the fact that
carboxyl groups can be both hydrogen donors and receptors, polymers formed by water-soluble acrylic monomers and their derivatives are among the most extensively used polymeric materials.

The polyacrylamide networks are relatively inert with low non-specific protein binding, and they reveal excellent biocompatibility in vivo.\textsuperscript{39,43} The polymerization mechanisms of the gel are well understood, and the gel properties can be easily manipulated to produce networks with a wide range of desired properties, including specific porosity, pore size and even specific mechanical strength.\textsuperscript{44}

Hjerten and co-workers demonstrated the synthesis of a polyacrylamide gel imprinted with a large number of proteins.\textsuperscript{40,41} They used acrylamide (AAm) and N, N-methylenebisacrylamide (MBAm) to produce a hydrogel with large pores. A series of proteins, including bovine hemoglobin (Hb), Cyt c and transferrin (Tf) and human growth hormone (HgH), RNase and horse myoglobin (Mb), were selected as target molecules. The synthesized bulk gel was granulated and packed into a column in which the template protein was washed out by acetic acid (HOAc) and sodium dodecyl sulfate (SDS). Protein was recognized by affinity chromatography in which various proteins passed through the column while only the target protein was selectively absorbed. As a result, all protein templates were absorbed specifically in their corresponding MIPed columns. The horse Mb-MIPed column even revealed an extremely high selectivity to distinguish its template from whale Mb, which has a very similar amino acid sequence and 3-D structure with horse Mb.

By using similar approaches, some other proteins such as BSA, Lyz, trypsin and Hb were recently imprinted in (mac)acrylic monomers synthesized polymers.\textsuperscript{37,45-48} It is
commonly accepted in these reports that steric factors and multiple point electrostatic interactions contribute to the overall strong interactions between the polymers and the template proteins, which consequently influence the capacity and selectivity of the imprints. However, a recent study on the effect of charge density to molecular recognition of imprinted hydrogel revealed that the interplay between hydrogel swelling, hydrogel-template electrostatic interaction and cavity recognition mechanisms remained difficult to interpret.49

Although the high template adsorption over non-imprinted counterparts was considered to be determined mainly by monomer-template interactions, other factors such as ionic strength, the extent of cross-linking and the easiness of template removal were also reported essential for discrimination efficiency of AAm-based imprints.50 In addition, mobility of large molecules in acrylate chemistry-based imprints requires a low degree of cross-linking, which may lead to reduced stability and mechanical properties.51 To overcome the problem, Guo et al. incorporated a protein imprinted polyacrylamide (PAA) gel with macroporous beads produced from chitosan, a natural biopolymer composed of D-glucosamine and D-acetyl-glucosamine.37 Both AAm and the template proteins (Hb) diffused to polymerize in the pores of chitosan beads. The polymer beads obtained from this approach were hydrophobic and demonstrated good mechanical strength as well as sufficient chemical stability. Moreover, by using BSA as the reference protein, the selectivity assay showed that the disassociation coefficient of Hb was more than 20 fold higher than that of BSA.

**Stimuli-sensitive hydrogel**

Stimuli-sensitive hydrogel is a type of hydrogel that can undergo reversible
volume transitions upon triggering by template binding or external stimuli such as temperature, pH, solvent composition, electric field, ionic strength, light or specific chemicals.\textsuperscript{52-60} The employment of template-sensitive hydrogel for imprinting protein was conducted by Chen and co-workers.\textsuperscript{61} As shown in Figure 2.1, the stimuli-responsive MIP, synthesized by using methacrylic acid (MAA) and AAm as functional monomers and N,N-methylenebisacrylamide (MBAm) as the cross-linker, responded to change in environmental stimuli such as temperature and solution ionic strength. In particular, a notable shrinkage of the gel occurred upon its binding with imprinted proteins (Lyz or Cyt c). The volume change was reversible: once the protein was released from the binding cavities, the hydrogel swelled back to its original size. The process was considered to be the result of sequential adaptation steps: the protein-responsive MIP may first adopt a state to favor the target binding in the presence of imprinted proteins. The electrostatic forces that dominate the interactions between functional monomer and protein drive the target protein partially fixing into the binding cavities. Subsequently, the flexibility of the polymer allowed the functional groups to align appropriately around the protein to form a complimentary structure, resulting in specific volume shrinking. While the mechanism of gel shrink is not fully understood, the report provides a good demonstration of “template-induced fit” property of the protein binding sites in synthesized MIP materials.

Tanaka and co-workers proposed in their work that molecularly imprinted hydrogels (heteropolymers) self-organized to form active sites with significantly different affinities to the target molecules in their swollen and collapsed states.\textsuperscript{53,55,56,62} Their work was also supported by Watanabe et al., who synthesized a temperature sensitive hydrogel
by copolymerizing N-isopropylacrylamide (NIPAm) and acrylic acid (AA) with a cross-linker in the presence of template norephedrine. The synthesized polymer underwent swelling at lower temperatures and collapsed at higher temperature. Upon exposure to the template molecules, the swollen gel at low temperature exhibited no further change, while the collapsed high-temperature hydrogel showed an increased swelling ratio, indicating that the template was memorized by the collapsed imprint rather than the swollen hydrogel. Moreover, norephedrine instead of reference molecule adrenaline induced the swelling, suggesting that the imprinted hydrogel was templatesensitive. The study also demonstrated that gel preparation conditions such as the polarity of the solvent also strongly affected the change of the polymer volume.

Before this study, Demirel et al. also used a pH / temperature sensitive hydrogel for imprinting BSA in poly (N-tert-butylacrylamide-coacrylamide/maleic acid) hydrogels. The template adsorption showed that pH, temperature and the initial BSA concentration influenced the BSA adsorption capacity for both non-imprinted and imprinted hydrogels. The maximum BSA binding to MIPed hydrogels was found at pH 5.0. The poly(TBA-co-AAm/MA) hydrogels interchange between a swollen phase at low temperature (5°C) and a collapsed phase at higher temperature (40°C). The protein binding conformation was unfavored with increased temperature. In spite of this, BSA-MIPed hydrogel exhibited higher template binding capability than non-imprinted polymers at all investigated temperature ranges (5°C - 40°C).

Moreover, the binding capacity was also found to relate directly with the concentration of template protein: the greater amount of template imprinted, the more BSA was captured in the polymers. The BSA adsorption capacity for the hydrogel
imprinted with 8.63 wt% BSA was as high as \(\approx 59\) mg of BSA per gram of dry hydrogel. In contrast, the adsorption for reference protein casein was considerably low, indicating high selectivity of the hydrogel to its template BSA rather than casein.

![Figure 2.1](image)

**Figure 2.1** Schematic representation of template-induced shrinking of the stimuli-sensitive hydrogel during the protein rebinding with its MIP (Modified from Ref.61)

**Sol-gel**

Sol-gel derived materials are another group of polymers that have been widely applied for protein imprints. They are colloidal suspensions of silica particles ("sol") that
are polymerized to form a solid gel. The starting materials (the precursors) used in the preparation of sol are usually inorganic metal salts or metal organic compounds such as metal alkoxides. The most commonly used precursors in the sol-gel process are tetramethylorthosilicate (TMOS) and tetraethylorthosilicate (TEOS). The hydrolysis of precursor reagents begins when they are mixed with water in the presence of an acid or a base catalyst, but the addition of a mutual solvent (usually alcohol) is not necessarily required. Hydrolysis of precursors leads to the formation of silanol groups (\(\equiv \text{Si}–\text{OH}\)), followed by progressive condensation reactions producing siloxane bonds (\(\equiv \text{Si}–\text{O}–\text{Si}\equiv\)), alcohol and water as by-products. During the sol-gel phase transition, the viscosity of the solution gradually increases as colloidal sol becomes interconnected to form a rigid, porous gel. Thus, sol-gel process provides a simple way to incorporate numerous organic, organometallic and biological molecules into a microporous matrix of sol-gel.

Further processing of sol or gel leads to sol-gel materials in different configurations. For example, as the viscosity of a sol is adjusted into a proper viscosity range, ceramic fibers can be drawn from the sol. A xerogel is formed when the solvent liquid in silica gel is removed and shrunk under ambient pressure. When solvent removal occurs under supercritical conditions, the network does not shrink and a highly porous, low-density material known as aerogel is produced. Heat treatment of a xerogel at elevated temperature effectively transforms the porous gel into a dense glass.

The physicochemical properties (e.g., average pore size, pore distribution, pore shape, surface area) of sol-gel derived materials depend mainly on sol-gel process parameters and the method with which the material is prepared. Those parameters include water-to-silane ratio, nature and concentration of precursor (hydrophilic / hydrophobic),
type of catalyst (acidic / basic), pH, nature of the solvent and ratio of solvent to precursor, temperature, humidity and aging (storage) conditions. Generally, low pH and low water ratio lead to denser materials with smaller average pore sizes, whereas high pH and high water preparations lead to more porous materials.\textsuperscript{65,67,68} Certainly, some of those factors will also be important to molecule recognition in polymeric matrices derived from sol-gel processes.

The earliest effort to prove that silane imprints could distinguish differences in the tertiary structure of proteins was made by Mosbach’s group.\textsuperscript{69} A boronatesilane was used as a functional monomer for interaction with the carbohydrate segment of the template glycoprotein transferrin (Tf), followed by polymerization within a silicate matrix on the surface of porous silica beads. High Performance Liquid Chromatography (HPLC) assays demonstrated that the Tf-imprinted silica beads had higher affinity for Tf than that for BSA (the control protein). Particularly, Venton and co-workers reported that the interaction between organo-functional side chains of silica-based monomers and the template proteins (urease or BSA) determined the complementary of protein binding pockets in their polysiloxane imprints.\textsuperscript{70,71} The monolithic gel prepared with 3-aminopropyl triethoxysilane (functional monomers) and cross-linker TEOS (1:3 molar ratio) showed preferential binding with target proteins. However, they failed to show any imprinting effects when two more closely related proteins labeled with \textsuperscript{125}I (\textsuperscript{125}I-Hb and \textsuperscript{125}I-Mb) were studied as the templates and the cross-binding controls, indicating that the assembly of monomers in the polymers may involve multiple factors. Zhang et al. used phenyltrimethoxysilane and methyltrimethoxysilane to prepare a HSA-imprinted thin film on the surface of a piezoelectric quartz crystal (PQC) Au-electrode modified with
thioglycolic acid.\textsuperscript{72} By using the piezoelectric quartz crystal impedance (PQCI) and the electrochemical impedance techniques, the Hb-imprinted PQC film showed that the binding capacity was affected by many factors such as salts, solvents, temperature and pH. In addition, Lee et al. recently showed that the potential binding sites on sol-gel derived macroporous polysiloxane scaffolds also related to the amount of the protein loaded into the sol before polymerization.\textsuperscript{73} The conclusion was drawn from their work on bulk imprinting of Lyz or RNase A via sol-gel processes, using TEOS as the cross-linker and \( \gamma \)-aminopropyltriethoxysilane (APTES) as the functional monomer. Up to 62\% of the loaded protein was surface accessible to the scaffolds. The imprinted scaffolds exhibited up to a threefold preference for binding with the template protein, even in the presence of similar-sized competitors. The difference in chemical properties of the templates and their consequent interaction with the polymer during embedding and imprinting of the biomolecules were considered significant to the results. Thus, the density of binding sites can be readily altered by changing protein loading.

Sol-gel materials have a combination of advantages over many other polymers for protein imprinting. First, the mild preparation conditions allow entrapped protein to retain much of its physical and chemical properties inside the polymer matrix. The compatibility with polar environments makes sol-gel material ideal for imprinting of biomolecules. Second, the rigid property of sol-gel cross-linking network favors retaining the shape of the cavities after removal of the templates. In addition, sol-gel glasses are electro-, photo- and chemically stable and can be optically transparent. The thickness, porosity and surface area of the material are easily controlled, while selectivity and diffusion for small template molecules are comparable and even better than acrylic
polymer-based imprinted materials.\textsuperscript{74,75}

Moreover, sol-gel reactions provide promising opportunities to develop organic-inorganic hybrid materials by incorporating organic components into inorganic polymers under mild conditions.\textsuperscript{76} There is a wide range of possibilities to obtain well-controlled properties of the materials by varying the chemical composition of the precursors and the ratio of inorganic to organic components.\textsuperscript{77} However, a significant obstacle for hybrid inorganic-organic sol-gel material is the chemical incompatibility between the two components. To apply the hybrid sol-gel polymer on protein-based MIPs is even more difficult. As a consequence, although a wide range of hybrid inorganic-organic sol-gel materials have been exploited as new sorbent phases for metal ions in the last 10 years, reports employing organic-inorganic hybrid sol-gels for protein imprinting are rare.\textsuperscript{78,79}

3-Aminophenylboronic acid (APBA) based MIPs

Poly(3-Aminophenylboronic acid) (poly-APBA) is an attractive matrix polymer for protein imprinting, not only because it can be easily synthesized by chemical oxidation of 3-aminophenylboronic acid (APBA) in mild environments but also due to the high content of the hydroxyl groups that mediate the reversible recognition of various bio-macromolecules via electrostatic and hydrogen interactions.\textsuperscript{80-82} In addition, poly-APBA can be grafted tightly onto the surface of materials such as polystyrene, glass and platinum by aromatic ring electron-pairing interactions, making it easy to integrate the polymer with sensor fabrication.\textsuperscript{82-86}

For instance, Rick and Chou fabricated protein imprinted electrodes by the cyclic voltammetric deposition of three layers of conductive polymers.\textsuperscript{87} An initial layer of
polypyrrole was used as the supporting polymer, which was coated with two layers of poly-APBA. Only the outer layer poly-APBA was deposited in the presence of the template protein Lyz or Cyt c. The target recognition of the MIPed polymer can be easily determined via current transmission reductions on the screen-imprinted platinum electrodes after polymer binding with non-conductive proteins. Rebinding of Lyz or Cyt c to the individual imprint showed a distinct two-phase binding profile, while the target protein captured on non-imprinted electrode exhibited a progressive binding that is typical for non-specific recognition. In the presence of 1 ppm protein, the current reductions for Lyz and Cyt c imprinted electrodes were 30.3% and 66.2%, respectively, whereas only 4.5% and 29.9% current reduction were obtained for the control electrodes, respectively. The same group also successfully applied quartz crystal micro-gravimetry (QCM) for demonstrating selectivity of Cyt c and Lyz imprints in poly-APBA films.88

By using similar QCM protocol, Turner et al. employed APBA as a protein imprint matrix for discriminating distinct isoforms of bovine BLG induced by temperature or fluoro-alcohols.89 As only a small portion of protein will be recognized, surface imprinting was usually considered unable to provide very high specificity. However, in this report, the MIPed APBA polymer showed moderate specificity for confirmation of the template instead of the non-template isoforms. In the meantime, greater changes of the protein secondary structure resulted in native BLG challenges to the imprint.

Although APBA chemistry is a fairly simple protocol that conforms to environmental constraints for protein imprinting, the interactions are highly dependent on the environment with respect to rebinding and MIP stability. Lu et al. recently
demonstrated that the adsorption of bovine Hb onto the poly-APBA grafted polystyrene micro-beads was pH dependent, where the strongest binding occurred at pH 6.0. The enhanced binding capability of the template was considered to be the result of increased electrostatic interactions between the positively charged Hb and the negatively charged poly-APBA at pH 6.0. Higher or lower pH led to repulsive electrostatic interactions. In addition, support material may also be associated with the stability of APBA imprints, as polystyrene (core-shell micro-beads) supported poly-APBA film possessed high stability for the template protein Hb, while poly-APBA grafted to glass rapidly lost its affinity to Hb.

**FUNCTIONAL MONOMERS FOR NON-COVALENT MIPs**

**Functional monomer-template interaction**

In a MIPed polymer, the individual binding site is formed by polycondensation of a monomer-template complex in the matrix (Scheme 1.1). For non-covalent MIPs, monomer-template interaction in the pre-polymerization complex is the same as monomer-target interaction during the rebinding process. Therefore, the affinity and selectivity of the binding sites are largely determined by the interaction between the functional monomer and the template. The more stable and strong the pre-polymerization complex is, the more selective the MIP is. In addition to the shape complementarity, the stability and the strength of the monomer-template complex are also largely determined by various non-covalent forces, including H-bond, hydrophobic interaction, ion pairing, electrostatic interaction and weaker dipole-dipole interactions. As those non-covalent forces are much weaker than covalent bonds, the overall selectivity and specificity of the imprinted receptor sites is determined by the sum of all interactions between the
functional monomers and the templates.

**Commonly used functional monomers**

In the last 20 years, many different functional monomers have been employed in non-covalent imprinting. Some commonly used examples classified according to their acid-base nature were presented in Figure 2.2. Among them, acidic monomers have been the most widely used in the literature. By far, the most successful monomers for non-covalent imprinting are carboxylic acid-based monomers, particularly methacrylic acid. It was first reported by Mosbach’s group and later was utilized for the imprinting of many templates such as 2,4-dichlorophenoxyacetic acid (2,4-D), nucleotides and cyclic peptides.\(^{90-93}\) In spite of the great tendency to dimerize, MAA was considered to interact strongly with the template by providing multiple ways of interaction (H-bond donor, H-bond receptor, dipole-dipole interaction, etc.), enhanced space-selectivity from its side groups (compared to AA) and the additional van der Waals interactions. In addition to MAA, 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPSA), 4-vinylbenzoic acid, AA, itaconic acid and (2-trifluoromethyl)acrylic acid (TFMAA) have all been applied in studies with different degrees of success.\(^{94-100}\) Some other acidic monomers, such as phosphates, have also been used for non-covalent imprinting via the formation of formal ion pairs under aqueous conditions.\(^{101}\) Among basic monomers, vinyl pyridine and the family of tertiary amino monomers have been the most commonly applied, as they have a strong tendency of forming \(\pi-\pi\) interactions with the templates. However, this reversely leads to greatly increased non-specific binding, which compromises the overall imprinting specificity and selectivity as compared to imprints formed by acidic monomers.\(^{102,103}\) Neutral monomers, such as AAm and its N-alkyl derivatives, methyl methacrylate and
Figure 2.2 Commonly used functional monomers in non-covalent molecular imprinting (Modified from Ref.102)
2-hydroxyethyl methacrylate (HEMA), have also been reported as effective monomers due to their strong ability to form H-bonds in non-polar media.\textsuperscript{37,104-107}

**Hydrophobic interaction in monomer-template complex**

The hydrogen bond has been shown to be the major factor for recognition in non-polar solvents.\textsuperscript{108-110} In aqueous conditions, the contributions of H-bond to recognition are screened by the presence of water, and alternative interactions such as electrostatic force and hydrophobic interaction become the major factors. For example, Chen et al. imprinted the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) to a co-polymer of 4-vinylpyridine (4-VP) and ethyleneglycol dimethacrylate. The binding of the template in aqueous solvents was mainly contributed by the accumulative effect of $\pi$-stacking and the electrostatic interactions. In another report, Zheng et al. prepared the imprint in polar organic media (acetonitrile) by a different combination of functional monomers, 4-VP with MAA or 4-VP with ACM. The first combination exhibited better recognition to the template sulfamethazine (SMZ) while the latter one has preferable binding with sulfamethoxazole (SMO). The recognition variance was considered to be largely due to the hydrophobic interactions between the template and the co-functional monomer.\textsuperscript{111}

In addition, hydrophobic interaction is more widely employed for polymeric recognition of proteins in aqueous media. The work of Zhang et al. provides an example that hydrophobic interaction is important for protein imprinting.\textsuperscript{112} In their report, the high specificity of Lyz imprint on the surface of a silica gel was largely due to the utilization of a functional monomer acryloyl-beta-cyclodextrin, which has a strong tendency to form inclusion compounds with hydrophobic guest through hydrophobic
interaction and hydrogen bonding. The functional monomer was also found effective on imprinting of bovine Hb by the same research group.

In most cases, protein recognition by the imprinted polymers is an accumulative effect in which multi-site hydrophobic interactions cooperatively work with other weak interactions such as electrostatic interaction or van der Waals force. For example, Chen et al. prepared a stimuli-sensitive imprinting for Lyz or Cyt c by using MAA and AAm as co-functional monomers, NIPAm as the major polymer and MBAm as the cross-linker. The greatly increased binding affinity for Lyz and Cyt c was found to be the cooperative contributions from hydrophobic interaction, hydrogen bonds and electrostatic forces.

**Optimization of pre-polymerization complex: ratio of functional monomer/cross-linker and of functional monomer/template**

In spite of the relatively simple strategy behind MIP technology, the selection of functional monomer and cross-linker, the ratio of monomer to template (M/T) or that of monomer to cross-linker (M/X) are often time consuming and hard to generalize for all substrates. The optimum value rarely corresponds to the complex stoichiometry predicted by spectroscopic analysis but is more based on a combination of experience and practice.

The employment of a single functional monomer is the most straightforward approach for non-covalent imprinting. Theoretically, the combination of different functional monomers is highly attractive because it may provide much greater binding affinity. However, in addition to the common requirements for optimization of a single monomer situation, the imprinting with various functional monomers requires that the sum of monomer-template interactions should be stronger than any interactions between
the monomers, which, in reality, is much more complicated and much harder to predict intuitively than for a single functional monomer. Hence, approaches based on the single functional monomer are by far the most widely reported for non-covalent imprinting.

As a minimum amount of cross-linkers are necessary for creating a polymer network rigid enough to sustain the binding sites, the ratio of M/X is limited mostly to the amount of the cross-linker. Once M/X is determined, the template concentration can then be optimized with respect to the functional monomer. Thus, the optimizations of M/X and M/T are usually determined empirically by evaluating several polymers with different formulations. For example, Sellergren and co-workers investigated the optimization of M/X for synthesis of an enantio-selective MIP for L-phehylalnine anilide (L-phe-an) by using MAA as the functional monomer and EGDMA as the cross-linker.\textsuperscript{116} MAA at molar ratio < 20\% increased the enantio-selectivity for L-phe-an versus D-phe-an, while loss of selectivity occurred when more than 20\% - 30\% MAA was applied.

To favor the formation of template-monomer complex in a pre-polymerization mixture, functional monomers are normally used in excess of template amount. Inadequate complexation with the template leads to poor yield of the recognition sites. However, too much functional monomer often results in high non-specific binding.\textsuperscript{116} Takateru et al. investigated the effect of the AA functional monomer on the binding specificity of Lyz-imprinted polymers on silica beads.\textsuperscript{98} The maximum binding specificity was produced when AA mixed with Lyz at 5:1 (molar ratio). The increased amount of functional monomer led to decreased binding, while no binding with Lyz occurred when AA was not polymerized in the polymer. The minimum ratios of functional monomer to template at 4:1 is rather common for non-covalent imprinting.\textsuperscript{117}
Mayes and Lowe tried the ratio of functional monomer (MAA) to template as high as 50:1, 150:1 and even 500:1 during the synthesis of a morphine imprinted polymer, yet the affinity of the recognition sites retained almost the same as imprint with 4:1 ratio of monomer/template.\textsuperscript{118}

**Functional monomer selection strategy**

The specificity, the selectivity and the function of imprinted binding sites are crucially determined by functional monomers to provide complementary interactions with templates and target molecules. In addition to the approaches based on thermodynamic calculations, the combination of several spectroscopic analyses such as Fourier Transform Inferred spectroscopy (FTIR), nuclear magnetic resonance (NMR) analysis and ultra-violet (UV) spectrophotometry have been more frequently applied on screening the appropriate functional monomers for rational design of MIPs.\textsuperscript{102,119,120}

NMR has been used mainly for the detection and the composition determination of monomer-template complex. For example, O'Mahony et al. applied NMR spectroscopy to identify the hydrophobic interaction between the template norephedrine (NEP) and the functional monomer 2,4-D in the pre-polymerization mixture.\textsuperscript{121} However, NMR cannot tell more accurately the exact position of a monomer to a template. Nuclear Overhauser Effect spectroscopy (NOE) is thus used due to its capability to display constraints between the near protons.\textsuperscript{102,122} FTIR is a technique that can be used for the detection of modified structures in a solution or at solid states. It has been employed in non-covalent imprinting to identify H-bonding formation and binding strength of hydrogen-bonded complexes due to the stretching frequency shift of hydroxyl or amino groups and carbonyl groups (respectively as hydrogen donor and acceptor).\textsuperscript{123}
The monomer screening based on spectroscopy approaches is usually time consuming in practice, as it requires the synthesis of certain amount of MIP. Thus, researchers attempt to use computational approaches or molecular modeling of the pre-polymerization complex system to assist in the screening of functional monomers from virtual monomer libraries. The strength of the interaction that each monomer is able to make with a template molecule is predicted by molecular modeling software. Subrahmanyam et al. developed an improved MIP for creatinine, in which the “optimized” functional monomer was selected by computer simulation. The computationally designed polymer was capable of distinguishing creatinine and creatine, while the non-computationally optimized MIP could not. Chianella et al. also applied the computational approach for virtual imprinting of an algal toxin microcystin-LR, which would have been difficult to do with a conventional approach due to the cost and toxic nature of the template. The affinity and the sensitivity of the MIPed polymer with the computationally selected functional monomers were even comparable to those of polyclonal antibodies.

In spite of several successful examples, the computer simulation of monomer-template interaction is not yet commonly applied, as the computational approach is based entirely on thermodynamic modeling for pre-polymerized complex, taking no account of the polymerization process. In addition, it still faces many challenges such as the extremely large computational workload required for such a complex system and the necessity to balance the monomer-template binding affinity and the selectivity.
PROTEIN SELECTIVE IMPRINTING APPLICATIONS

MIPs have been widely recognized as a very promising methodology for preparation of tailor-made materials with selective adsorption. They have been intensively used for investigation and development of sensors, catalysts or sorbents for solid-phase extraction and stationary phases for liquid chromatography and so on. Although most of the examples are from the imprinting of small molecules, protein-selective MIPs have shown significant development in recent years. Major application areas for protein imprints in analytical separation and biosensor will be discussed.

Analytical separation

By far the most popular application for molecular imprinted materials is for analytical separation and purification of target molecules from mixed fluids or solutions. This application involves the synthesis of a monolithic polymer that has to be crushed and sieved to obtain particles of the desired size and distribution. The method is fast and simple in its practical execution and is widely reported in the literature for many different templates. For instance, Tong et al. developed a Hb-imprinted polyacrylamide-agarose column by entrapment of soft gel in the pores of rigid, inert agarose beads.\(^\text{39}\) The gel formed was cut into pieces, and the agarose beads were freed from the surrounding polyacrylamide gel by stirring the mixture. The chromatographic properties of these gels, including the protein binding capacity and the flow rate, were demonstrated fourfold higher than non-template imprinted polymers (NIPs). Recently, Qin et al. presented a facile method for Lyz-imprinted polymer beads.\(^\text{127}\) By surface initiated radical polymerization (SIP), Lyz was copolymerized with AAm and MBAm in aqueous media.
and grafted on the surface of mesoporous chloromethylated polystyrene beads (MCP beads) containing dithiocarbamate iniferter (initiator transfer agent terminator). The polymeric beads were packed into a column for chromatographic separation. The results showed that the Lyz-MIPed column exhibited good capability for separating Lyz from the control proteins, including bovine hemoglobin, bovine serum albumin, ovalbumin and cytochrome c.

Some alternative polymerization strategies for the preparation of better analytical-grade imprinted materials have been proposed in the literature during recent years. Among many published papers, molecular imprinted solid phase extraction (MISPE) is one of the fastest-growing applications. However, the method is predominantly applied to the imprinting of small molecules; the reports on protein-imprinted MISPE are rare. In two closely related fields, affinity capillary electrophoresis and electro-chromatography (CEC), protein imprinting has been conducted mostly during the last decade. For example, Takátsy et al. copolymerized iron-free or iron-saturated transferrin with AAm and MBAm for preparation of imprinted artificial antibodies. The gel was granulated and freed from the selectively adsorbed template protein (as the antigen in this technique). By using the free-zone electrophoresis in a revolving capillary, the study revealed that it cannot distinguish between iron-free and iron-saturated transferrin due to their similar mobility in gel, whereas significant differences in the mobility of selective gel granules were observed depending on their interaction with iron-free or iron-saturated transferrin. By using a similar analysis approach, the same group also demonstrated a high selectivity of polyacrylamide-based imprinting for human and bovine Hb species.
Recently, Denizli and co-workers presented a remarkable example of using protein-based MIPs for virus detection from biological fluids.\textsuperscript{130} They imprinted a hepatitis B surface antibody (HBSA) in a monolith column prepared from poly (hydroxyethyl methacrylate-N-methacryloyl-l-tyrosine methyl ester) (PHEMAT), and N-methacryloyl-l-tyrosine methyl ester was used as the functional monomer for interaction with HBSA. After the polymer column was dried, ground and sieved, particles 20-63 µm in size were obtained. Adsorption studies were performed on HBSA and anti-hepatitis A antibody-positive human plasma. Competitive adsorptions of HBSA, total anti-hepatitis A antibody (AHAA) and total immunoglobulin E (Ig E) were investigated for demonstrating the selectivity. The adsorption capability of the particle imprints for HBSA was 18.3 times more than that for AHAA, and 2.2 times more than that for Ig E.

Denizli’s group also fabricated a protein imprint for efficient and cost-effective purification of Lyz from egg white.\textsuperscript{131,132} In this work, Lyz template was imprinted into macroporous cryogels, which were formed in frozen solutions of monomeric or polymeric precursors.\textsuperscript{133} \(\text{Cu}^{2+}\) was complexed with metal-coordinating monomer N-Methacryloyl-(l)-histidinemethylester (MAH), and the free radical polymerization of Lyz-MIPed cryogels was initiated by N,N,N,N-tetramethylene diamine (TEMED) in an ice bath. The relative selectivities of the Lyz-MIPed cryogel for target protein over BSA and Cyt c were 4.6 and 3.2 times greater than non-imprinted cryogel, respectively. Moreover, Lyz purity desorbed from Lyz-MIPed cryogel can be well maintained (~ 94%) and recovery rates up to 86% attained. In particular, the binding capacity retained good stability for target rebinding even after many adsorption–regeneration cycles, indicating that the Lyz imprints could be applied as an efficient, simple and cost-effective
alternative for large scale Lyz separation.

**Biosensors**

As an analytical device, a chemical sensor is typically composed of a molecular recognition element and a transduction scheme to detect and analyze binding. Molecularly imprinted materials can be utilized as artificial sensing elements to create biosensors by combining with various physicochemical transducers.

The mainstream of small-molecule imprinting uses bulk monolith; their incorporation into sensor platforms is not trivial. In contrast, protein-based MIPs are predominantly surface imprinted; thus the incorporation of a protein-MIPed film / membrane with sensor surfaces has become more and more popular for the development of novel types of biosensors in recent years. Generally, the surface modification of a sensor by protein imprints is achieved by spin coating or using other surface grafting techniques to deposit a thin layer of protein-imprinted polymer on the surface of a sensor chip. After gel polymerization and the removal of the template, an imprinted sensor surface for recognition of specific protein analytes is thus formed. For example, Rick and Chou deposited a thin layer of poly-APBA onto the gold surface of quartz crystal microbalance (QCM) using Lyz or Cyt c as the templates. The modified electrode demonstrated a good selectivity for the template proteins over competitive proteins, including albumin and Mb. A remarkable achievement of the experiment was that the MIPed polymer could recognize Lyz and Cyt c at the same time when they were used simultaneously as templates. In another report by the same group, cyclic voltammetric deposition was applied to provide more control of the protein-imprinted polymerization process on a screen-printed platinum support. As a result, enhanced reproducibility of the
thin polymer film with defined conductivity was also obtained.\textsuperscript{87}

Recently, some groups explored the fabrication of protein-MIPed biosensors for specific detection of virus proteins and even whole viruses. Ramanaviciene prepared a polypyrrole-based MIP for direct detection of the bovine leukemia virus (BLV) glycoprotein gp51 (gp51).\textsuperscript{136} The polymer was prepared as a surface coating onto a platinum electrode in a potassium chloride solution. By using pulsed amperometric detection (PAD), the research group monitored the binding, washing and rebinding of the protein over several cycles and found that the recognition and the affinity degraded with each repeat. Recently, Tai and co-workers fabricated an artificial receptor sensor for specific recognition of dengue virus by epitope imprinting on the surface of a quartz crystal microbalance (QCM) chip.\textsuperscript{137} Although an epitope of the dengue virus NS1 protein, pentadecapeptide, was employed as the protein template, the modified sensor surface exhibited increased polymer affinity toward both pentadcapeptide and NS1 protein. Moreover, quantitative detection of the dengue virus protein was achieved. In the presence of NS1 protein, the modified QCM chip even demonstrated a comparable frequency shift to the control chips immobilized with monoclonal antibodies.

Optical sensors fabricated by imprinting low molecular weight targets onto fluorescence responsive MIPs have been widely reported, but very few have been reported for protein imprinting.\textsuperscript{138} Recently, impressive work has been done by Bright’s group, who described protein imprinted xerogels with integrated emission sites (PIXIES).\textsuperscript{139} The polymer was first prepared by protein imprinting in sol-gel. After the template was washed away, the imprinted gel was treated with a non-covalent complex of the template protein and an arylazide-derivatized luminophore (BODIPY). Once the
protein binds with imprinted xerogels, the lumimophore reporter groups were also guided into the binding cavities. Upon UV irradiation, the azide functionality converted into an extremely reactive nitrene and inserted into the xerogel. Thus, the presence of specific protein in binding sites led to the changes of the imprints’ fluorescence. Bright prepared various PIXIES films constructed from 16 silane monomer formulations, including n-octyltrimethoxysilane (C8-TMOS), bis(2-hydroxyethyl)-aminopropyl-triethoxysilane (HAPTS), TEOS and APTES. By using ovalbumin as the template and fluorescent arylazide-derivatized BODIPY as the luminophore, all silane formulations showed increased fluorescence upon rebinding with ovalbumin, among which the highest emission was about 10 times higher than the control.

The protein-responsive PIXIES films also demonstrated strong selectivity, as its responses to competing proteins (HSA and chemically modified ovalbumin) was over 200-fold smaller. In addition, a remarkable property of the PIXIES system is the high stability of the quantum yield of BODIPY upon changes in pH or solvent polarity. This property excludes the possibility of increasing fluorescence by microenvironment changes induced by analyte binding, which has been reported in other sol-gel fluorescence imprints.\textsuperscript{140,141} The efficiency of the fluorescent protein imprinting system was further demonstrated by using human interleukin-1 alpha (IL-1a) and beta (IL-1b) as templates. The two interleukins, 27% of which are homologous, can be identified by the highly responsive PIXIES, demonstrating the high potential of MIP-based optical sensors for sensitive, high-throughput peptide / protein screening.
Fluoro-alcohol-induced protein structure transitions

Alcohols were recognized to affect the structure and function of peptides and proteins with research dating three decades.\textsuperscript{142,143} Alcohol-based cosolvents have also been used for many years to denature proteins and to stabilize induced structures in peptides. The ability of aliphatic alcohols to denature proteins increases with their effective methylene chain length (i.e., pentanol $>$ butanol $>$ propanol $>$ isopropanol).\textsuperscript{144,145} However, the protein denaturation potency of alcohols decreases beyond a certain chain length (typically about 8-12 methylene groups, depending upon the system), which is called the cut-off phenomenon.\textsuperscript{146-149}

Among many alcohols, fluoro-substituted alcohols such as TFE and HFIP have been used as the most effective co-solvents for evoking structure changes in protein and peptides.\textsuperscript{150} It has been well known that TFE and HFIP partially denature protein native structures and induce alpha-helix in numerous proteins and their fragments (Table 2.2). For this reason, the fluoro-alcohols have been widely used for transformation of protein into molten globule intermediate, stabilization of various intermediate or dissolving of amyloid aggregates.\textsuperscript{151-157} Moreover, conformational changes of nucleic acid induced by the fluoro-alcohols have also been reported.\textsuperscript{158,159} Between the two fluoro-alcohols, TFE usually requires higher concentrations than HFIP to obtain the same extent of structure effects, suggesting the greater efficiency of HFIP over TFE.\textsuperscript{154,160-163}

TFE with higher concentrations was reported to be useful in dissolving protein aggregates, while lower concentrations of TFE promoted the beta structures via forming
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Alcohol</th>
<th>Induced Structure</th>
<th>Method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp-cage</td>
<td>TFE</td>
<td>long-lived fluoro-peptide complexes (42% v/v)</td>
<td>intermolecular H-1{F-19} NOE</td>
<td>164</td>
</tr>
<tr>
<td>S-peptide of ribonuclease A</td>
<td>TFE, HFIP</td>
<td>enhanced helical propensity (1 M HFIP, 3 M TFE)</td>
<td>CD spectra</td>
<td>161</td>
</tr>
<tr>
<td>melittin</td>
<td>HFIP</td>
<td>alpha-helix (35% v/v)</td>
<td>comparative molecular dynamics simulations</td>
<td>165</td>
</tr>
<tr>
<td>TC-peptide of thermolysin</td>
<td>TFE, HFIP</td>
<td>enhanced helix propensity (40% TFE, 27.4% HFIP, v/v)</td>
<td>CD spectra</td>
<td>161</td>
</tr>
<tr>
<td>silk model peptide</td>
<td>HFIP</td>
<td>alpha-helix (HFIP as the solvent)</td>
<td>$^{13}$C- NMR</td>
<td>166</td>
</tr>
<tr>
<td>octapeptide</td>
<td>TFE, HFIP</td>
<td>alpha-helix (both as the solvents)</td>
<td>two-dimensional inferred spectroscopy</td>
<td>162</td>
</tr>
<tr>
<td>Alzheimer’s beta-peptide</td>
<td>HFIP</td>
<td>alpha-helix (&gt; 20%, v/v), soluble amyloid-beta aggregates (2% HFIP, v/v)</td>
<td>CD spectra, electron microscopy AFM, NMR</td>
<td>167,168</td>
</tr>
<tr>
<td>Nα-acylated heptapeptide amide</td>
<td>HFIP</td>
<td>alpha-helix (HFIP as the solvent)</td>
<td>CD spectra, X-ray diffraction</td>
<td>169</td>
</tr>
<tr>
<td>islet amyloid polypeptide</td>
<td>TFE</td>
<td>amyloid fiber, beta-sheets (60% TFE, 10% HFIP, v/v)</td>
<td>CD spectra, fluorescence spectroscopy, TEM</td>
<td>170</td>
</tr>
<tr>
<td>22-residue peptide of microglobulin</td>
<td>TFE, HFIP</td>
<td>amyloid-like fibril (20% TFE, 10% HFIP, v/v)</td>
<td>Atomic force microscopy</td>
<td>160</td>
</tr>
<tr>
<td>Protein</td>
<td>Alcohol</td>
<td>Induced Structure</td>
<td>Method</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------</td>
<td>-------------------------------------------------------------</td>
<td>-------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>papain</td>
<td>TFE, HFIP</td>
<td>alpha-helix and molten globule intermediate (13% HFIP, 30% TFE, v/v)</td>
<td>CD spectra, fluorescence spectroscopy</td>
<td>154</td>
</tr>
<tr>
<td>succinylated Con A</td>
<td>TFE, HFIP</td>
<td>alpha-helix (60% TFE, 30% HFIP, v/v)</td>
<td>CD spectra, fluorescence spectroscopy</td>
<td>163</td>
</tr>
<tr>
<td>pea lectin</td>
<td>HFIP</td>
<td>alpha-helix (12% - 30%, v/v)</td>
<td>CD spectra, fluorescence spectroscopy</td>
<td>171</td>
</tr>
<tr>
<td>beta-lactoglobulin</td>
<td>TFE, HFIP</td>
<td>alpha-helix (0.6 M HFIP, 10-20% TFE, v/v)</td>
<td>CD spectra</td>
<td>172,173</td>
</tr>
<tr>
<td>human serum albumin</td>
<td>TFE, HFIP</td>
<td>alpha-helix (~ 0.5M HFIP, ~ 1 M TFE)</td>
<td>CD spectra, fluorescence spectroscopy</td>
<td>156</td>
</tr>
<tr>
<td>bovine alpha-lactalbumin</td>
<td>HFIP</td>
<td>molten globule state (0.5 M -0.75M)</td>
<td>DSC, CD spectra, fluorescence spectroscopy</td>
<td>155</td>
</tr>
<tr>
<td>prion protein (PrP)</td>
<td>TFE, HFIP</td>
<td>alpha-helix, modified PrP amyloids (fluoro-alcohols as the solvents)</td>
<td>FTIR, fluorescence spectroscopy, Electron microscopy</td>
<td>174,175</td>
</tr>
<tr>
<td>cytochrome c</td>
<td>HFIP</td>
<td>alpha-helix, molten-globule state (2.5% - 7.5% HFIP, v/v)</td>
<td>CD spectra, fluorescence spectroscopy, small-angle X-ray scattering</td>
<td>153</td>
</tr>
<tr>
<td>lentil lectin</td>
<td>TFE, HFIP</td>
<td>alpha-helix (10% -30% HFIP, 60% TFE, v/v)</td>
<td>CD spectra</td>
<td>157</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Induced Structure</td>
<td>Method</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------------------------------------------------</td>
<td>-------------------------------------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>silk fibroin protein</td>
<td>alpha-helix and 3(10)-helix (fluoro-alcohols as the solvents)</td>
<td>$^{13}\text{C}$-NMR, CD spectra</td>
<td>176</td>
<td></td>
</tr>
<tr>
<td>apomyoglobin</td>
<td>molten globule intermediate (4% HFIP, v/v)</td>
<td>fluorescence spectroscopy, hydrogen/deuterium exchange mass spectrometry</td>
<td>177</td>
<td></td>
</tr>
<tr>
<td>creatine kinase</td>
<td>alpha-helix (2.5% - 5% HFIP, v/v)</td>
<td>CD spectra, fluorescence spectroscopy, enzyme activity assays</td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>alpha-synuclein</td>
<td>beta-sheet aggregates (20% TFE, v/v) alpha-helix (50% TFE, v/v)</td>
<td>CD spectra</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>acylphosphatase</td>
<td>amyloid fibril (25% TFE, v/v)</td>
<td>CD spectra, fluorescence spectroscopy</td>
<td>179</td>
<td></td>
</tr>
<tr>
<td>acidic fibroblast growth factor</td>
<td>amyloid fibril (&lt;10%, v/v) aggregates dissolved (&gt;10%, v/v)</td>
<td>CD spectra, fluorescence spectroscopy</td>
<td>152</td>
<td></td>
</tr>
<tr>
<td>alpha-chymotrypsin</td>
<td>amyloid-like aggregates (~35% TFE, v/v)</td>
<td>CD spectra, fluorescence spectroscopy, TEM, FTIR</td>
<td>180,181</td>
<td></td>
</tr>
<tr>
<td>tau protein</td>
<td>alpha-helical aggregates (20% TFE, v/v)</td>
<td>CD spectra, AFM, FTIR, electron microscopy</td>
<td>182</td>
<td></td>
</tr>
<tr>
<td>bovine serum albumin</td>
<td>alpha-helical aggregates (TFE &gt; 6.2M)</td>
<td>CD spectra, fluorescence spectroscopy</td>
<td>183</td>
<td></td>
</tr>
</tbody>
</table>
amylloid fibril in several models.\textsuperscript{152,180,181,184} Pallares et al. reported that alpha-chymotrypsin, an all beta protein, aggregated into amyloid-like structures in the presence of TFE, where the maximal amount of fibrilary aggregation was formed at 35\% (v/v) TFE.\textsuperscript{185} The CD data suggested that the protein exists in an extended beta-sheet conformation. In contrast, higher TFE concentration (> 40\%) led to reduced aggregation propensity. Additionally, TFE also induced the beta-sheets formation in acylphosphatase (a small alpha / beta protein) and acidic fibroblast growth factor (an all beta-sheet protein).\textsuperscript{152,179}

It is well known that neurodegenerative diseases are related with the ready formation of fibril aggregation by amyloidogenic proteins. Indeed, the propensity to self-assemble into amyloid structures is a common property of polypeptides.\textsuperscript{186} Hence, a lot of investigations have been done on exploring the capability of the fluoro-alcohols on disrupting amyloid formation, or dissolving / reassembling the amyloid fiber.\textsuperscript{160,166,167} For example, Tomaselli and co-workers used HFIP to induce reversible conformation transition of Alzheimer’s beta-(1-42) peptide.\textsuperscript{167} In this study, beta-conformations were mainly maintained in 1\% HFIP-water mixture (v/v) while the formation of alpha-helices was observed when HFIP content was increased over 20\%. In addition to beta-sheets, structures containing beta–hairpins have also been characterized in several reports.\textsuperscript{187,188}

Generally, protein aggregation is associated with the formation of beta structures; however, alpha-helical aggregates have also been mediated by fluoro-alcohols.\textsuperscript{189-191} In a recent study, TFE favored the assembly of tau protein into alpha-helical aggregates, the process of which involved the arrangement of preformed alpha-helices into coiled-coils.\textsuperscript{182} Sen et al. also reported that TFE enhanced the alpha-helical conformations
with a simultaneous increase of aggregation in alkaline unfolded BSA, while the introduction of aliphatic alcohols such as methanol, ethanol and 2-propanol led to the formation of beta-sheet-like structure.\textsuperscript{183} Moreover, HFIP and TFE have been reported to be able to partially recover the activity of acid-denatured proteins. For example, Naseem et al. studied the reaction activity of lentil lectin with dextran and bromelain in the presence of HFIP and TFE.\textsuperscript{157} The fluoro-alcohols stabilized an acid unfolded (at pH 1) intermediate of lectin by inducing alpha-helices. The acid-induced activity loss of lectin with bromelain was found partially recovered by both fluoro-alcohols, in which a 30\% regain in enzyme activity was observed with HFIP as compared to a 15\% regain with TFE.\textsuperscript{154} The same group also observed that 13\% (v/v) HFIP and 30\% (v/v) TFE induced the regain of the enzymatic activity for acid-denatured papain (pH 2), accompanying the formation of a compact “molten globule like” intermediate with near-UV CD spectrum approaching native-like spectral features.\textsuperscript{154}

**Mechanism for fluoro-alcohol-induced protein structural transitions**

Although various activities of the fluoro-alcohols on protein conformations have been observed, the detailed mechanism has not yet been well understood, nor has the reason why the two fluoro-alcohols have superior effectiveness over other alkanols in evoking the structure transitions been explained in a satisfactory manner. Uversky et al. observed that a protein structure transition is highly correlated with the relative dielectric constant of several organic solvents.\textsuperscript{192} Indeed, an excellent correlation is seen between $\Delta G$ of helix formation and the solvent dielectric in non-halogen alcohols. As lower solvent polarity around the protein weakens the hydrophobic interaction that stabilizes the compact native structure of protein, it has been suggested that the reduced solvent
polarity caused by alcohol molecules determines their activity on unfolding a protein. However, the correlation is poor in the case of the fluoro-alcohols. TFE and HFIP are all polar solvents with high ionizing power. The relative dielectric constant of them are both much lower than water but are comparative with other alkanols that have much weaker protein effects (Table 2.3). Hence, factors other than the lowered solvent polarity must play more important roles for the significantly enhanced structure inducing efficiency of the fluoro-alcohols.

The hydroxyl moiety of alkanol makes the alcohol molecule not only weakly acidic but also a good hydrogen donor instead of a proton acceptor. It has been suggested that alcohols induce protein structure transitions by directly binding or interacting with a carboxyl group rather than an amide group of proteins, although the binding mechanism has not been well understood and the indication on properties of the binding sites are inconsistent in the literature. Early research by Abraham et al. has suggested that the actual binding site should include a hydrogen bond acceptor group, but Eyring et al. proposed that alkyl alcohol binds with hydrophobic moieties in protein. By using $^{13}$C NMR multiplet relaxation technique, Nicolai et al. demonstrated recently that ethanol bound to three out of five hydrophobic binding sites for cis-parinaric acid in BSA via ethyl group instead of ethanol hydroxyl group. In addition, some other studies suggested that hydrophobic binding sites may be spatially limited to the size of the alcohol molecule, as the anesthetic effects of alkanols decreased beyond a certain chain length.

Due to the strong electronegativity of F groups, TFE has been suggested to interact with polypeptide simultaneously as a proton donor and acceptor: the hydroxyl
group preferentially binds with a protein backbone carbonyl oxygen group as a proton donor, while F group acts as a hydrogen acceptor forming H-bonds with proton donor groups on protein. As a consequence, the solvent exposure of the amide is minimized and the intra-polypeptide hydrogen bonding of the amide group is favored, leading to increased helical propensities.

Table 2.3 Chemical properties of water and various alcohols (Adopted from Ref. 202).

<table>
<thead>
<tr>
<th></th>
<th>Dielectric constant*</th>
<th>Dipole moment*</th>
<th>Refractive index*</th>
<th>pKa§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>78.5</td>
<td>1.84</td>
<td>1.333</td>
<td>15.7</td>
</tr>
<tr>
<td>Methanol</td>
<td>32.6</td>
<td>1.70</td>
<td>1.329</td>
<td>15.5</td>
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<tr>
<td>Ethanol</td>
<td>24.3</td>
<td>1.69</td>
<td>1.361</td>
<td>15.9</td>
</tr>
<tr>
<td>Propanol</td>
<td>20.1</td>
<td>1.58</td>
<td>1.385</td>
<td>16.1</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>18.3</td>
<td>1.58</td>
<td>1.377</td>
<td>~25</td>
</tr>
<tr>
<td>Butanol</td>
<td>17.1</td>
<td>1.66</td>
<td>1.399</td>
<td>~16</td>
</tr>
<tr>
<td>TFE</td>
<td>26.6</td>
<td>2.52‡</td>
<td>1.291</td>
<td>12.4¶</td>
</tr>
<tr>
<td>HFIP</td>
<td>~10†</td>
<td>-</td>
<td>1.275</td>
<td>9.3¶</td>
</tr>
</tbody>
</table>

* These values were obtained from the CRC Handbook of Chemistry and Physics unless otherwise noted.
† This value for HFIP (hexafluoroisopropanol) was estimated in Ref. 204.
‡ This value for TFE (trifluoroethanol) was reported in Ref. 200.
§ Except where noted, the pKa values were taken from Ref. 205 and Ref. 206.
¶ These values were determined by Dyatkin et al.

Hydrophobicity of alcohols has been suggested essential to their activities on polypeptide structures, as longer chain aliphatic n-alcohols (e.g. propanol, butanol) are much more potent than methanol. The higher efficiency of HFIP and TFE clearly suggests that the presence of –CF₃ may contribute to fluoro-alcohol induced structure transitions. Rajan et al. proposed in their study that the increased hydrophobicity, which arises from both hydrocarbon groups and multiple F atoms in the fluoro-alcohols, plays an essential role in denaturing protein tertiary structures and inducing the formation of helical structures. Hirota et al. also demonstrated in their work that the hydrocarbon
groups contributed positively to the effectiveness of alkenols, while an increased number of hydroxyl groups led to decreased structure-inducing effects.\textsuperscript{208,209}

In spite of these explanations, the remarkable structure-inducing effects of HFIP over TFE are far stronger than expected. The self-aggregation property of the two fluoro-alcohols that was demonstrated by X-ray scattering spectroscopy studies was suggested to be essential for HFIP or TFE induced great structure transitions in proteins.\textsuperscript{210-215} HFIP has a high tendency to self-aggregate to form micelle-like assemblies in aqueous solution. HFIP aggregation in water produces clusters with a Stokes radius of about 24 Å, and the extent of clustering reaches to the maximum at ~ 30\% (v/v). TFE also tends to form micelle-like clusters but to a lesser extent with an apparent Stokes radius of about 6 Å.\textsuperscript{210-215} The formation of aggregation leads to further reduced solvent polarity around the protein. In addition, coordination of a second and a third molecule in the fluoro-alcohols increases the polarization of the terminal hydroxyl groups, which consequently leads to enhanced H-bond donor activity.\textsuperscript{212,215,216} As a result, the effects of HFIP and TFE are largely enhanced.

Taken together, the fluoro-alcohols interact with protein both as hydrogen bond donors and acceptors, whose binding are stabilized by hydrophobic interactions. The much remarkable activities of HFIP over TFE on inducing protein structure changes are associated with its stronger hydrophobicity, the greater H-donor and H-acceptor activities associated with incorporation of multiple F atoms as well as the higher tendency of HFIP to aggregate.

**From fluoro-alcohol to fluoro-silane**

Although HFIP and TFE have pronounced activities on inducing protein structure
transitions, the fluoro-alcohols are highly corrosive and non-polymerizable. These properties prevent them from being employed for MIP synthesis. 3F and 7F as the structure analogues of TFE and HFIP, respectively, can be easily polymerized in silica gel via sol-gel reactions. Therefore, 3F and 7F were proposed in this report as the functional monomers to induce protein conformational transitions in TEOS gel. The synthesis of fluoro-modified TEOS sol-gel and the influences of 3F / 7F on structure transitions of template proteins are demonstrated in detail in following chapters.

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CHAPTER 3

BOVINE SERUM ALBUMIN ENCAPSULATION AND CONFORMATION IN FLUORO-MODIFIED SILICA GELS*

Abstract: Silica gels capable of inducing defined conformational changes in proteins were synthesized by polymerization of either 3,3,3-trifluoropropylmethoxysilane (3F) or (3-heptafluoroisopropoxy)propylethoxysilane (7F) with tetraethylorthosilicate (TEOS). Bovine serum albumin (BSA) and fluorescein isothiocyanate conjugated BSA (FITC-BSA) were encapsulated in the gels. The hydrolyzed TEOS sol mixing with 0.01 M pH 8 hepes buffer at a ratio of 1:3 (sol:buffer) was selected as the optimal condition for rapid synthesis of rigid and optically transparent gels. These 1:3 gels doped with 10% 3F (3F/TEOS, mol%) or with 5% 7F (7F/TEOS, mol%) were as transparent as pure TEOS gels, whereas 10% 7F modification resulted in a turbid gel containing micron-sized 7F aggregates that excluded encapsulated FITC-BSA. In contrast, FITC-BSA was homogenously dispersed in the pure TEOS and 10% 3F modified TEOS gels. Water contact angle increased from 19.9º ± 2.6º on pure TEOS gel to 85.8º ± 3.4º on 5% 3F-modified gels and to 105.4º ± 1.1º on the 5% 7F gels. However, 10% 7F modification reversed the hydrophobicity trend by reducing the contact angle to 55.2º ± 4.2º.

The fluorospectroscopic assays on the fluoro-silane-BSA interactions showed that either hydrolyzed 3F monomers or 3F copolymerized up to 10% in TEOS induced a concentration-dependent fluorescence reduction for 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS), a lipophilic probe that has high affinity with the hydrophobic pockets of

* Coauthored by Yun Peng and David W. Britt
proteins. The 3F-induced probe emission reduction may be interpreted as strong BSA-3F interactions excluding ANS-protein interactions. In contrast, hydrolyzed 7F increased the probe fluorescence, while 5% 7F in TEOS gel quenched the emission, suggesting that solution 7F facilitates ANS binding to BSA by exposing hydrophobic pockets on the protein; however, upon condensation into a TEOS gel, this effect is lost, possibly due to steric constraints in the gel. These findings demonstrate the advantages of 3F over 7F as a functional monomer for TEOS gel modification in terms of retaining gel transparency and increasing hydrophobicity (and consequently stability against capillary-induced collapse) while maintaining a uniform distribution of encapsulated protein. Encapsulated protein appears to be stabilized by 3F binding to hydrophobic domains or clefts on the protein as evidenced by exclusion of the lipophilic probe, 1,8-ANS.

INTRODUCTION

The sol-gel process as a route to form inorganic glasses at relatively low temperatures has been known for over a century, but only until recent years has the encapsulation of biochemically active macromolecules such as protein, nucleic acid, peptide and even whole cells into sol-gel derived materials matured into its own discipline.\(^1\text{-}^4\) The synthesis of materials by sol-gel process generally involves two steps: the hydrolysis of alkoxide precursors (sol) yielding silanols, followed by the condensation polymerization to form gels. Due to the mild conditions of sol-gel chemical reactions, and the significant amount of water contained within the hydrated glass, the entrapped bio-species are able to maintain their activities in sol-gel derived silica gels.\(^5\text{-}^6\) Moreover, the use of various polymer additives, redox modifiers or organo-silane provides versatile ways to incorporate functional groups into silica gels. A wide variety of
enzymes, antibodies and functional proteins has been entrapped into a diverse range of
sol-gel derived materials,\textsuperscript{5,7-10} yielding applications ranging from the development of
chemical and biochemical sensors and high-throughput screening assays to controlled
drug-release matrices and biocatalysis.\textsuperscript{3,6,11-19}

Fluorine doping in silica glass has been widely applied to improve the physical
properties of polymeric materials, particularly the optical properties, as fluorine reduces
the refractive index without any evident absorption in the transmission region of
silica.\textsuperscript{20-22} Moreover, fluorine enhances thermal and chemical stabilities, fosters the silica
glass structural relaxations, and significantly reduces dielectric constant.\textsuperscript{20,21,23,24} Organic
fluorine has been widely described as having a distinct influence on protein structure and
stability in aqueous system.\textsuperscript{25,26} However, little is known about the interaction of protein
with fluoro-containing sol-gel monomers, and even less has been done on the influences
of polycondensed fluorine on structure and bioactivity of gel-encapsulated protein.

In this study, fluoro-alkyl modified silica gel was synthesized using the
hydrophobic silane molecules, (3-heptafluoroisopropoxy)propylethoxysilane (7F) and
3,3,3-trifluoropropylmethoxysilane (3F) as “functional” monomers to cross-link with
tetraethylorthosilicate (TEOS) in the sol-gel processes. Here, “functional” refers to
monomers that exhibit distinct protein affinity or activity, which for the fluoro-silanes
includes a strong influence on protein secondary structures. The synthesis conditions of
the sol-gel reactions were optimized to obtain optically transparent silica gels and
uniformly dispersed protein. The interaction of the fluorine containing silane monomers
with the template protein bovine serum albumin (BSA) was monitored spectroscopically
by fluorescence of protein tryptophan residues and 1-anilinonaphthalene-8-sulfonic acid
(1,8-ANS). The probe is essentially non-fluorescent in aqueous media, where binding with hydrophobic domains on protein leads to remarkably enhanced fluorescence (quantum yields approximately 0.7),\textsuperscript{27,28} making it a sensitive indicator for protein conformational transitions.\textsuperscript{29-33}

**EXPERIMENTAL**

**Materials**

3,3,3-Trifluoropropylmethoxysilane (3F, >95% purity, MW = 218.3, d = 1.14 g cm\(^{-3}\)) and tetraethylorthosilicate (TEOS, > 99% purity, MW = 208.3, d = 0.94 g cm\(^{-3}\)) were purchased from Gelest, Inc (Morrisville, PA). 3-(Heptafluoroisopropoxy)propyltriethoxysilane (7F, > 95% purity, MW = 309.4, d = 1.4 g cm\(^{-3}\)) was synthesized by SynQuest Laboratories, Inc. (Alachua, FL). 2,2,2-trifluoromethanol (TFE, 99% purity), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, > 98% purity), 1M hepes buffer (pH 7 and pH 8), bovine serum albumin (BSA, MW = 66,400) and fluorescein isothiocyanate conjugated BSA (FITC-BSA, MW = 67,000) were purchased from Sigma-Aldrich (St. Louis, MO). The 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS, > 95% purity) was bought from Molecular Probes, Inc. (Eugene, OR).

HFIP and TFE were diluted with 0.01 M pH 2.5 hepes buffer at volume ratios ranging from 2% to 25% (corresponding to 0.19 M - 2.39 M for HFIP and 0.28 M - 3.48 M for TFE). BSA stock solutions (15.6 μM) were prepared by dissolving 20 mg BSA in 20 ml 0.01 M pH 2.5 or pH 8 hepes buffer. FITC-BSA (15 μM) was prepared by dissolving 5 mg FITC-BSA in 5 ml hepes buffer (0.01 M pH 8). The stock solution of 1,8-ANS (334 μM) was prepared at two pH values by adding 1 mg 1,8-ANS in 10 ml 0.01 M pH 2.5 or pH 6.5 hepes buffer containing 40% ethanol, then placed in an
ultrasonication bath (Fisher Scientific, Hanover Park, IL) to facilitate dissolution. Both BSA and 1,8-ANS solutions were stored at 4°C, and the probe solution was used for up to three days.

**Gel preparation and optimization**

The hydrolyzed silane sol was prepared by following the protocol used by many groups, where 4.5 ml TEOS precursor was mixed with 1.4 ml double distilled water (ddH₂O) and 0.1 ml 0.1 M HCl. The mixture was ultra-sonicated in an ice bath for 30 minutes, followed by stir-mixing (600 rpm) in an ice bath for about five to six hours until the mixture became clear and monophasic.

To obtain a fluorinated TEOS sol, 3F or 7F were mixed with TEOS at 10% or 5% (mol%) before addition of H₂O and HCl (5% fluoro-silane/TEOS was prepared by mixing 195 μl 3F or 225 μl 7F with 4.3 ml TEOS; 10% fluoro-silane/TEOS was prepared by mixing 356 μl 3F or 411 μl 7F with 4.11 ml TEOS). Following the same ultrasonication and mixing process for pure TEOS sol, the primarily hydrolyzed fluoro-silane/TEOS sol was kept at -20°C for at least seven days to facilitate controlled hydrolysis at low pH. The starting pH of the sol mixtures are around 2.5, as the pH favors a maximum hydrolysis rate for aqueous silicate while the overall condensation rate for TEOS sol-gel reaction is maximized around pH 7. Hence, the condensation of the TEOS gel can be induced by increasing buffer pH.

The gelation rate and the optical transparency of TEOS gel vary with the pH of 0.01 M hepes buffer used to initiate condensation and the ratio of hydrolyzed silane sol to this buffer. To explore the optimal condensation conditions, the hydrolyzed silane was rapidly stir-mixed (600 rpm) with 0.01 M hepes buffer (pH ranging from 4 to 8) at
various volume ratios (4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4). A 1 ml aliquot of the condensation mixture was pipetted into each well of a 24-well plate (Costar, Corning Inc., NY) for observing gel formation. In addition, additional condensation mixtures with various ratios of soliane sol to hepes buffers (0.01 M) at pH 7 or pH 8 were prepared for encapsulating 4.5 μM BSA (300 μl 15 μM BSA was contained in every 1 ml condensation mixture). A 200 μl aliquot of the protein / condensation mixture was pipetted into the well of a UV-transparent 96-well plate (Costar, Corning Inc., NY) for spectroscopic assay on the gel turbidity by absorbance at 600 nm. The gelation time is defined as the earliest time the solution exhibits gel properties, which is taken when flow is no longer visibly observed in tilted wells. Before each assay, the freshly formed silica gels in well plates were aged at 4°C for one to three hours with the plate lid on to prevent drying and possible gel cracking.

**Fluorescence microscopy**

To visualize the protein in cured silica gels, the hydrolyzed TEOS sol prepared from the above protocol was rapidly stir-mixed (600 rpm) with 0.01 M pH 8 hepes buffer at three volume ratios of 1:3, 1:1 and 3:1. FITC-BSA stock was added in each mixture to yield a final concentration of 4.5 μM. Around 10 - 15 μl of the mixed sol was dispensed on a glass slide pre-cleaned with ethanol and distilled water, then carefully covered with a glass coverslip to avoid entrapment of air bubbles. Once the coverslip was held in place due to the gel formation (about five minutes), a thin layer of nail polish was brushed to seal the perimeter of the coverslip to the glass to prevent water evaporation and gel cracking. The slides were stored at 4°C until analysis.

An inverted fluorescence microscope (Nikon Eclipse TE2000-S) with 100 W
Xe-lamp was used to study the distribution of FITC-BSA in silica gel. The images were captured using a 10× objective and a three-second integration time by a Nikon DXM1200 color CCD camera supported with ACT-1 (Nikon) software from a PC with frame-grabber.

**Fluorescence spectroscopy**

The steady-state fluorescence intensity for protein intrinsic and extrinsic fluorophores was monitored by a microplate spectrofluorometer (BioTek Instruments, Inc., Winooski, VT). The fluorescence of tryptophan residues was excited at 290 nm, and the emission was collected at 330 nm. The excitation and emission wavelengths for 1,8-ANS were set at 360 nm and 460 nm, respectively.

**Protein and probe fluorescence in pH 2.5 hepes buffer.** The stock solution of hydrolyzed silanes (1.2 M) was prepared by mixing 2.66 ml TEOS, 2.30 ml 3F and 2.65 ml 7F with pH 2.5 hepes buffer (0.01 M) at 7.34 ml, 7.70 ml and 7.35 ml, respectively. The mixtures were stir-mixed (600 rpm) in an ice bath until the silanes were miscible with water (five to six hours for TEOS and 7F, two to three hours for 3F). The hydrolyzed silanes was diluted by 0.01 M pH 2.5 hepes buffer and mixed with BSA (15 μM, pH 2.5) and 1,8-ANS (334 μM, pH 2.5) to monitor the fluorescence of 1,8-ANS. The concentrations of the hydrolyzed silane in the sample mixtures ranged from 0.1 M to 0.6 M; ANS and BSA concentration in each mixture was 90 μM and 4.5 μM, respectively. For assays on Trp emissions, the sample mixtures were prepared the same way as the above but without the addition of 1,8-ANS. Following 30 minutes equilibrium at room temperature, 200 μl of each sample mixture was pipetted into the wells of a 96-well plate (Costar, Corning Inc., NY) to monitor the fluorescence. The diluted fluoro-alcohols HFIP
and TFE were used as the benchmark controls. Each data point presented is the average of at least four independent measurements, and appropriate blank data (from corresponding protein-free sample) was subtracted.

1,8-ANS fluorescence in fluoro-modified TEOS gel. The condensation mixture was prepared by stir-mixing (600 rpm) 250 μl HCl-hydrolyzed silane sol (TEOS, 3F/TEOS or 7F/TEOS) with 300 μl BSA (15 μM, pH 8) and 450 μl hepes buffer (0.01 M, pH 8.0). A control mixture without protein was prepared in parallel. A 200 μl aliquot of the protein and control mixtures were immediately pipetted into two columns of a 96-well plate. The pH of the condensation mixtures was around 6.5, and the gels formed after five to ten minutes standing at room temperature. The freshly prepared gels were aged at 4°C for one to three hours, followed by rinsing each gel with 150 μl hepes buffer (0.01 M pH 6.5) to attain a uniform pH and to remove the alcohol products from the polycondensation. Next, 100 μl diluted 1,8-ANS (180 μM, pH 6.5) was added on top of each gel to diffuse and bind with the protein in the gel. The probe fluorescent emission was monitored at a 20- or 30-minute interval of the diffusion until the fluorescence reached a plateau. Before each assay, the plate was placed upside down for two to three minutes to drain the remnant ANS solution not adsorbed into the gel. Each data point presented is the average of eight independent measurements, where the blank (protein-free gel) values were subtracted from each sample.

Gel surface hydrophobicity

The water contact angle was measured with a VCA optima digital contact angle instrument (AST products, Billerica, MA) on the surface of intact silica gels using 250 μl sessile drops of ddH₂O. The gels were prepared in the same way as in the
flurospectroscopy assays (with 4.5 μM BSA entrapped). Due to the fragile nature of TEOS gel without fluoro-modification, slight disturbances of the gel monolith may cause gel breaking. To obtain intact surface for measurements, about 800 μl of the condensation mixture was immediately dispensed in a shallow polystyrene well (2 cm × 2 cm × 0.08 cm) (Corning Inc., Corning, NY). The plate was then placed at 4°C to allow for a controlled rate of polycondensation (approximately 15 - 20 minutes) without gel cracking. The contact angles were then collected on flat areas of the gel surface, and each data point presented is the average of at least ten independent measurements.

RESULTS AND DISCUSSION

TEOS sol-gel synthesis

Increasing temperature or raising the pH of the hydrolyzed silane sol can trigger the polycondensation step of the sol-gel process. In our reactions at room temperature, the polycondensation rate of TEOS and fluorosilane was governed by the pH of 0.01 M hepes buffer that mixed with the hydrolyzed silane as seen in Figure 3.1. These results show rapid condensation for pH 7 and 8 buffers, whereas below pH 6, condensation was very slow, taking at least 5 hours for gelation (see Figure A.1). With the addition of 0.1 M pH 8 hepes buffer, gels formed within 20 to 60 minutes, depending on the volume of condensation buffer added. An optimal range of sol:buffer ratios between 4:1 and 1:4 was determined at pH 8 (Figure 3.1). At higher and lower ratios, condensation still occurred but did not lead to stable gels due to the mixture either being too concentrated or diluted, respectively (data not shown).

The turbidity of the gels formed by pH 7 and pH 8 condensation buffers were presented in Figure 3.2. As shown from the results, with the addition of pH 8
condensation buffer, the diluted buffer not only favored faster gelation but also increased the gel optical transparency, whereas under the same dilutions, the pH 7 condensation buffer led to the increasing of gel turbidity. The gels formed by sol:buffer (pH 8) at 1:3 and 1:4 ratios were colorless and clear, while these ratios at pH 7 were visibly opaque. The gel prepared at a 1:4 ratio of sol:buffer (pH 8) were less rigid as compared with the gels formed from less diluted buffer. Thus, to rapidly synthesize a robust and highly transparent TEOS gel for protein encapsulation, the TEOS sol was mixed with 0.01 M pH 8 hepes buffer at the volume ratio of 1:3. Gel stability and protein properties were further tuned by addition of fluorosilanes as described next.

Figure 3.1 Gelation time at neutral and slightly basic pH values as a function of the ratio of hydrolyzed TEOS sol to 0.01 M hepes condensation buffer. When hydrolyzed TEOS was mixed with 0.01 M pH 7 hepes buffer at ratios higher than 1:1, the condensation was delayed beyond five hours (solid symbols). The standard deviations are within ± 8%, and the error bars are omitted for clarity (n = 4).
Figure 3.2 Dependence of gel turbidity on the ratio of hydrolyzed TEOS sol to condensation buffer for silica gels prepared by 0.01 M pH 7 and pH 8 hepes buffer. 4.5 μM BSA was encapsulated in each gel. Error bar represents the standard deviation of each data point (n = 5).

Figure 3.3 depicts the gel optical transparency for pure TEOS gels and fluoro-silane modified TEOS gels that formed by mixing the hydrolyzed silane sol with 0.01 M pH 8 hepes buffer at a 1:3 volume ratio. As shown from the results, when hydrolyzed TEOS preloaded with either 5% or 10% (mol%) 3F, the mixed-gels had no resolved turbidity changes. Neither did BCA encapsulation influence the gels optical transparency. However, doping TEOS with 3F monomers promoted the rates of hydrolysis and gel polycondensation. By stir-mixing on an ice bath, 3F/TEOS sol became monophasic after approximately three hours, whereas pure TEOS sol took almost six hours to be miscible with water. Similarly, the polycondensation process for 3F/TEOS sol took about 15 minutes, which is approximately 2/3 of the time for pure TEOS to form a gel (data not shown). The enhanced condensation may be anticipated for fluorosilanes
due to the high electro-negativity of the multiple fluorines that will tend to place a partial positive charge on the silane reaction center, thus favoring nucleophilic attack by water during hydrolysis. Condensation of the fluorosilane sol is anticipated to be accelerated compared to TEOS, as the electron depleted silanols of 3F is readily reduced to form Si-O-Si bonds. These constituent effects must be balanced against possible steric hindrance of the bulky fluorines and possible self-aggregation.

While the accelerated gelation induced by doping TEOS with up to 10% fluoro-silane did not affect gel turbidity for 3F, 10% 7F modification led to a significant increase of the gel turbidity. Moreover, the encapsulation of 4.5 μM BSA in 10% 7F-TEOS gel increased the turbidity up to about 4.5 times higher than the corresponding protein-free gel (Figure 3.3). Fluorescence microscopy was then used to further examine

![Figure 3.3](image-url)

**Figure 3.3** The turbidity of TEOS silica gel doped with either 5% or 10% (mol%) ratios of 3F or 7F. ○: Gel encapsulated with 4.5μM BSA. ◇: Gel formed without BSA entrapment. Error bar represents the standard deviation for each data point (n = 8).
the distribution of protein in the gels as a function of fluoro-silane content and ratio of sol to condensation buffer as described next.

**Protein distribution in fluoro-silane/ TEOS gel**

The fluorescence microscope images in Figure 3.4 depict the distribution of 4.5 \( \mu \text{M} \) FITC-BSA in silica gels prepared by mixing TEOS or fluoro-silane/TEOS sols with 0.01 M pH 8 hepes condensation buffer at three volume ratios (3:1, 1:1 and 1:3). The images reveal that protein distribution is somewhat similar for all gels prepared at the same ratio of sol:buffer (columns). For all 3:1 gels, the protein appeared as bright aggregates, suggesting precipitation of protein during condensation in these concentrated systems (Figure 3.4, left column). As the volume of condensation buffer was increased, protein aggregation decreased during condensation (Figure 3.4, middle and right columns). Two factors contribute to the protein aggregation with decreasing condensation buffer volume. First, a tighter gel network arises for increasing sol:buffer ratios. Second, as the sol:buffer ratio was increased from 1:3 to 1:1 to 3:1, the gel pH decreased from 6.2 to 5.6 to 5, respectively. The extent of protein aggregation corresponds to the proximity of gel pH to the isoelectric point (pI) of the entrapped BSA (pI = 4.8). The greatest FITC-BSA aggregation occurred in pH 5 gel, while the least / no aggregates were observed in pH 6.2 gel.

Although fluorescent aggregates were not evident for any of the 1:3 ratios, dark spots of uniform size were seen in the fluorescence images of TEOS gel modified with 10% 7F (Figure 3.4, panel c), indicating that 7F monomers may self-aggregate into compact domains that exclude proteins.
Figure 3.4 Fluorescence microscopy reveals the strong influence of the volume ratio of hydrolyzed silane to 0.01 M pH 8 hepes condensation buffer on the distribution of 4.5 μM FITC-BSA in pure TEOS or 10% fluoro-silane modified TEOS gels. Protein aggregates occur for all gels at sol:buffer ratios of 3:1 and 1:1 (left and middle columns). For a 1:3 ratio, protein aggregation is not apparent (right column); dark spots different from FITC-BSA aggregates are only observed for gel containing 10% 7F (panel c), attributed to 7F silane self-aggregation into compact domains.

Surface hydrophobicity of fluoro-silane/TEOS gel

The surface hydrophobicity of 3F and 7F modified gels was analyzed by monitoring the water contact angle on the gels. Table 3.1 shows that the water contact angle on pure TEOS gel surface is small ($\theta_{H_2O} = 19.1^\circ \pm 2.6^\circ$), verifying the hydrophilic properties of the silicate gel matrix. Incorporation of the fluoro-silane functional monomers greatly increased the gels hydrophobicity. Water contact angles on the gels doped with 5% and 10% 3F were both around 85° ($\theta_{H_2O} = 85^\circ \pm 3.4^\circ$), while 5% 7F
modification resulted in further increased surface contract angle \( \theta_{H_2O} = 104.5^\circ \pm 2.6^\circ \), indicating the stronger hydrophobicity of 7F over 3F. However, for the gel doped with 10% 7F, the water contact angle dropped to 53.0° ± 5.7°, suggesting a heterogeneous distribution (aggregation) of 7F silane over 3F in the gel, as also evidenced by the fluorescence microscopy data of the gels doped with 10% 7F in Figure 3.4 and increased gel turbidity for the 10% 7F gels in Figure 3.3.

**Table 3.1** Sessile water contact angles \( \theta_{H_2O} \) on surface of TEOS gels containing 4.5 μM BSA and modified with 3F or 7F at 5% and 10% (fluoro-silane/TEOS, mol%), respectively. Each value is the average of at least 10 independent measurements.

<table>
<thead>
<tr>
<th>Gel precursors</th>
<th>( \theta_{H_2O} ) (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEOS</td>
<td>19.9° ± 2.6°</td>
</tr>
<tr>
<td>5% 3F/TEOS</td>
<td>87.0° ± 3.6°</td>
</tr>
<tr>
<td>10% 3F/TEOS</td>
<td>84.8° ± 3.1°</td>
</tr>
<tr>
<td>5% 7F/TEOS</td>
<td>105.4° ± 1.1°</td>
</tr>
<tr>
<td>10% 7F/TEOS</td>
<td>55.2° ± 4.2°</td>
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</tbody>
</table>

**Hydrolyzed fluoro-silane interaction with BSA**

The fluorospectroscopic assays on the emissions of intrinsic Trp and extrinsic 1,8-ANS fluorophores were applied to elucidate the mode of BSA interactions with hydrolyzed fluoro-silanes in hepes buffer (0.01 M, pH 2.5) and with fluoro-silanes copolymerized into TEOS gels using pH 8 condensation buffer. The results for the solution studies are shown in Figure 3.5, where a rapid quenching of BSA intrinsic fluorescence was induced by hydrolyzed 3F at low concentration (< 0.1 M); however, no
further emission reduction occurred at concentrations above 0.2 M. Additionally, hydrolyzed 3F monomers significantly decreased the steady-state fluorescence intensity of 1,8-ANS mixed with BSA, indicating 3F may have a substantial influence on the protein structure. Alternatively, a competitive binding between 1,8-ANS and 3F for hydrophobic sites on BSA may exist. The benchmark structure-inducing fluoro-alcohol, HFIP, also quenched Trp and 1,8-ANS fluorescence, but it required significantly higher concentrations ($\times 3 - 5$) to induce the same quenching levels observed for 3F (Figure 3.5).

In contrast, hydrolyzed 7F exhibited a distinct interaction with the protein. It enhanced the emission intensities of both fluorophores, suggesting that 7F may induce protein conformation transitions that lead to decreased water access but increased 1,8-ANS binding to the hydrophobic binding sites on BSA. In addition, TFE and hydrolyzed TEOS monomers showed little influence on emissions of Trp and 1,8-ANS, verifying the inert TEOS-BSA interaction and the much weaker structure-inducing activities of TFE than HFIP.

**Fluoro-silane interaction with BSA in gel**

The interaction of hydrolyzed fluoro-silane monomers with protein were investigated to provide these functional monomers maximum degrees of freedom to explore the protein topography, bind to hydrophobic domains and form possible hydrogen bonds with the protein, which would be preserved upon introducing TEOS and raising the buffer pH to induce condensation.

To understand the interaction of the 3F and 7F fluoro-silane functional monomers with the protein once polycondensed in TEOS gels, 1,8-ANS was allowed to diffuse into the gels and the fluorescence emission was followed with time as shown in Figure 3.6.
Figure 3.5 The fluorescence intensity of protein Trp residues and the probe 1,8-ANS as a function of the concentration of hydrolyzed silane, TEOS, 3F and 7F, or fluoro-alcohols, HFIP and TFE. (a) Trp emission is significantly quenched by 3F and HFIP but not by TFE or 7F. The TEOS control has little effect as expected. (b) The fluorescence intensity of 90 μM 1,8-ANS is quenched most significantly by 3F, while 7F enhances 1,8-ANS emission. [BSA] = 4.5 μM in 0.01 M pH 2.5 hepes buffer. The standard deviations of the data are within ± 2% (n = 4), and the error bars are omitted for clarity.
Figure 3.6. Increased fluorescence of 90 μM 1,8-ANS after a two-hour diffusion into 3F or 7F modified silica gels containing 4.5 μM BSA. (a) 3F modification led to a concentration-dependent decrease of 1,8-ANS fluorescence. (b) Reduced probe fluorescence was maximum for 5% 7F, whereas self-aggregation for 10% 7F resulted in greater 1,8-ANS fluorescence, suggesting fewer protein-7F interactions due to 7F clustering into protein-free domains (see Figure 3.4, panel c). The standard errors for all data were within ± 5%, and the error bars are omitted for clarity (n = 8).
From this kinetic analysis, the probe binding with BSA in each gel reached equilibrium after diffusion for approximately two hours. In comparison with the probe fluorescence in pure TEOS gel, all fluoro-modification induced the reduction of the probe emission intensities. 3F led to a dose-dependent fluorescence decrease: 5% 3F and 10% 3F modification, respectively, led to 25% and 65% loss of the probe emissions, indicating a reduced 1,8-ANS accessibility to binding sites on BSA in the 3F-modified TEOS gels. In contrast, the 7F interaction with protein is more complex. While 5% 7F modification led to a 48% loss of the probe fluorescence, a diminishing effect was observed for the 10% 7F gel, where only a 25% reduction in probe emission was observed. The result here again indicates the self-aggregation of 7F when doped at 10% in the TEOS gel.

**Structure-inducing activities of 3F and 7F**

ANS is almost non-fluorescent in water; however, adsorption into hydrophobic sites in proteins significantly enhances the probe fluorescence (quantum yields around 0.7),\textsuperscript{27,28} as a change in protein hydration or ANS accessibility to water leads to profound change of the fluorophore emission. The probe is therefore widely used as a sensitive indicator for protein structure transitions.\textsuperscript{36-39} Although the fluoro-silanes are hydrophobic, 1,8-ANS in hydrolyzed 3F and 7F solutions was as non-fluorescent as that in water in absence of BSA (data not shown). Hence, 3F or 7F induced 1,8-ANS fluorescence transitions are unlikely due to the fluoro-silane-ANS interactions, but they may have resulted from the protein structure transitions induced by fluoro-silane-BSA interactions that consequently influenced the hydrophobic binding with 1,8-ANS.

Based on our results, 3F influenced the probe fluorescence in a way like HFIP but exhibited significantly stronger fluorescence-quenching effects, pointing toward greater
structure-inducing activities of 3F compared to this benchmark fluoro-alcohol (Figure 3.5). In contrast, 7F exhibited complicated interactions with BSA, where hydrolyzed 7F showed opposite effects compared to polycondensed 7F on the fluorescent emissions of 1,8-ANS. Indeed, at 5% concentration in TEOS gel, 7F was a stronger quencher of 1,8-ANS fluorescence than 3F. It is unclear why 7F promotes 1,8-ANS binding to BSA in solution, yet once 7F is condensed with TEOS, binding of this probe to BSA is restricted.

Studies employing X-ray scattering and dynamic light scattering have shown that the multiple F atoms in TFE or HFIP cooperatively promote clustering of the alcohol molecules.\textsuperscript{36-38} This clustering was also proposed as an important factor for interpretation of the high potential of HFIP on inducing conformational transitions in investigated proteins.\textsuperscript{26,37} Considering the constituent similarity of F atoms between the two fluoro-alcohols and the two fluoro-silanes (3F vs. TFE and 7F vs. HFIP), fluoro-silane monomers with a bulky hydrophobic group may tend to self-aggregate to form larger clusters than one with a less bulky group. Thus, hydrolyzed 7F monomers added to TEOS at 10% molar ratios may locally bind with each other and form large aggregates during the polycondensation, which was evident as dark spots under fluorescence microscopy and consequently leads to decreased optical transparency of the synthesized silica gel (Figure 3.3 and 3.4, respectively). The reversal in the surface hydrophobicity trend (Table 3.1) and 1,8-ANS fluorescence for 10% 7F modified gels (Figure 3.6) also supports the aggregation or sequestration of 7F at higher concentrations. Additionally, although 3F and TFE have identical trifluoro moieties, TFE has almost no influence on probe fluorescence at investigated concentrations (< 0.6 M), indicating the structure-inducing
activity of the trifluoro-silane, inferred through strong quenching of Trp and 1,8-ANS fluorescence emission, is not merely related with the fluoro-groups. The alkyl-silanol groups clearly play an important role that is not compromised upon polycondensation of 3F with TEOS. These results support the use of 3F as a functional monomer for protein encapsulation in sol-gels.

**CONCLUSIONS**

Fluorine containing TEOS gels were synthesized by doping 3F or 7F in TEOS sol then raising the pH to induce gelation. A 1:3 ratio of sol to pH 8 condensation buffer resulted in rapid formation of transparent and stable gels. This ratio also led to a homogeneous distribution of FITC-BSA in TEOS gels with up to 10% 3F or 5% 7F fluoro-modification. Both 10% 3F and 5% 7F modifications significantly increased gel surface hydrophobicity without compromising transparency. The 5% 7F-modified gel was the most hydrophobic ($\theta_{5\%7F} = 105.4^\circ$ vs. $\theta_{3F} = 85.8^\circ$); increasing 7F content to 10% decreased hydrophobicity ($\theta_{10\%7F} = 55.2^\circ$) and significantly elevated gel turbidity due to 7F self-aggregation in the TEOS gel. Fluorescence microscopy images of FITC-BSA distribution in 10% 7F/TEOS gel (1:3 of sol:buffer) provided direct evidence for 7F self-aggregation in the form of micron-sized dark domains that excluded FITC-BSA, which was otherwise uniformly present in the gel.

The interaction of BSA with the hydrolyzed 3F or polycondensed 3F was followed through intrinsic and extrinsic fluorophores that underwent significant quenching in a manner consistent with HFIP-induced conformational changes in the protein. These data suggest that both hydrolyzed 3F and polycondensed 3F monomers induced shifts in BSA structure. Of particular significance is that 3F was effective at
concentrations three to five times lower than HFIP, which is the benchmark protein secondary structure-inducing agent. Retention of this activity upon copolymerization of the fluoro-silanes in TEOS gels supports their use as functional monomers for both encapsulating and enhancing the secondary structure of proteins.

An alternative scenario of competitive binding between 3F and 1,8-ANS for hydrophobic pockets in protein may also exist and cannot be excluded from the data presented here. In contrast, 7F monomers exhibited complex interaction with BSA. Hydrolyzed 7F enhanced the probe binding with BSA, while polycondensed 7F reduced the fluorescent emissions of 1,8-ANS in the gel, paralleling the behavior of polycondensed 3F in TEOS. Thus, 3F is demonstrated as a superior functional monomer than 7F for fluoro-modification of TEOS gel in terms of the gel optical transparency, the distribution of the protein and the interaction with gel-encapsulated protein.

REFERENCES


CHAPTER 4

TRIFLUOROSILANE INDUCES STRUCTURAL TRANSITIONS
IN BETA-LACTOglobulin: A FUNCTIONAL
MONOMER FOR CATALYTIC PROTEIN

CONFORMATIONAL IMPRINTING*

Abstract: Misfolded protein can self-aggregate due to destabilization of alpha-helices and the simultaneous formation of beta-sheets. The beta-sheet mediated loop-sheet polymerization results in formation of fibrils and insoluble plaques that result in protein conformational diseases. The development of molecularly imprinted polymers (MIPs) capable of inducing protein structural transitions, in particular the re-formation of alpha-helices from a “pathogenic” beta-sheet isoform, is thus of great interest. Here, the efficiency of (3,3,3-trifluoropropyl)trimethoxysilane (3F) as a functional monomer for synthesis of a protein conformational imprint in tetraethylorthosilicate (TEOS) sol-gels was investigated. The interaction of 3F with the template protein beta-lactoglobulin in a pre-polymerization complex led to conformational shifts in the protein as inferred by significant changes in the fluorescence emission intensities of protein tryptophan residues and the lipophilic probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS). Circular dichroism confirmed that these shifts corresponded to a 3F-induced alpha-helical structure. The activity of 3F was compared with the well-known alpha-helix-inducing agents 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and 2,2,2-trifluoroethanol (TFE). The 1,8-ANS fluorescence data revealed that hydrolyzed 3F led to the formation of a molten globule-like intermediate at just 1/3 the concentration of HFIP (0.2 M vs. 0.6 M). The non-radiative fluorescence resonance energy transfer (FRET) between the tryptophan

*Co-authored by Yun Peng, Nicholas W. Turner and David W. Britt
residues and 1,8-ANS showed that 0.2 M 3F-induced FRET was three times greater than that by 0.6 M HFIP, indicating the strong structure-inducing activity of the trifluoro-silane. Additionally, 3F-induced BLG conformation transitions were retained upon polymerization of 3F in a TEOS matrix, validating the use of 3F as a biologically functional monomer readily incorporated into sol-gels.

**INTRODUCTION**

Molecularly imprinted polymers (MIPs) are synthetic materials that contain template molecule-induced binding cavities that are spatially and chemically complementary to the template molecules. Since molecular imprinting technology was developed three decades ago, MIPs have been intensively explored as materials with high selectivity for numerous compounds, including cholesterol, monosaccharides, amino acids and drugs, leading to applications in multiple fields such as drug delivery and drug discovery, analytical separation, biosensing and antibody/receptor mimics.

For imprinting of biomacromolecules such as a protein or an enzyme, however, MIP development has been more tedious due to the intrinsic limitations of macro-templates exhibiting complex surface structure and chemistry, conformational flexibility and poor solubility in non-polar solvents. In spite of these challenges, the generic, robust and cost-effective properties of MIP technology, as well as the great application potentials for protein-selective MIPs in the fields of biomaterials, biosensors and biomedical technology, have made protein-based MIPs very attractive research topics for both scientific and industrial communities.

The three-dimensional structure of protein is of vital importance for its function in
a living organism. Misfolded proteins may result in more than a non-functional molecule. In many cases, misfolded proteins associate directly with the onset of “protein conformational diseases” such as Alzheimer’s, Parkinson’s, cataracts and Crutzfield-Jakob.\textsuperscript{13,14} The molecular basis for almost all protein conformational diseases involves the formation of aggregates caused by destabilization of alpha-helices and the simultaneous formation of beta-sheets.\textsuperscript{15} Substances that favor the stabilization of alpha-helical structure or inhibit the formation of beta-sheets can be potentially useful to recover the structure of a misfolded protein. Therefore, the development of a MIP that can induce protein conformational transitions, particularly the stabilization or the formation of alpha-helices, is of great interest. It is proposed in our research that such a catalytic protein conformational imprint (CPC-MIPs) can be applied for analysis of proteins linked with conformational diseases or even can serve as a catalyst to correct misfolded proteins.

An important process in MIP technology is the formation of a template-monomer complex prior to adding cross-linking agents and initiating polymerization. The interaction in this pre-polymerization complex determines the selectivity, the specificity and the functionality of an imprinted polymer. Hence, to further MIP technology, a library of functional monomers is essential to optimize the selectivity and the stability and to fine-tune the monomer-template interaction and the monomer and cross-linking agents.

In spite of diverse functional monomers that have been employed for protein-selective MIPs, monomers that can guide conformational transitions for target proteins have not been studied. Fluoro-alcohols such as 2,2,2-trifluoroethanol (TFE) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) have been well known for inducing
alpha-helices in various proteins such as pea lectin, papain, succinylated Con A and cytochrome c.\textsuperscript{16-19} However, they are highly corrosive and non-polymerizable, precluding their use in MIPs. A fluoro-silane (3,3,3-trifluoropropyl)trimethoxysilane (3F) is thus proposed as the polymerizable structure analogue of TFE; it is capable of serving as a bioactive functional monomer for fabrication of protein conformational imprints via sol-gel processes.

In this report, the 3F-protein interactions in the pre-polymerization complex and in TEOS gels were investigated to validate the structure-inducing activity of 3F. Beta-lactoglobulin (BLG), a model protein frequently used for protein structures studies, was employed as the template protein. The 3F-induced protein conformational changes were demonstrated by fluorospectroscopic assays on fluorescent emissions of protein tryptophan residues (Trp) and the probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS), which has a high affinity with protein hydrophobic pockets and is used almost exclusively as a standard for detection of the protein molten globule intermediate.\textsuperscript{16,17,19-22} Moreover, the non-radiative fluorescence resonance energy transfer (FRET) between the two fluorophores was also applied for demonstrating the extent of protein conformational changes. The efficiency of the structure-inducing activity of 3F was assessed by using HFIP and TFE as the benchmarks. The interactions between BLG and tetraethylorthosilicate (TEOS) and n-propyltrimethoxysilane (nPM), respectively, as the sol-gel matrix monomer and the propyl substitution of TEOS were also employed to interrogate the molecular composition contributions of the trifluoro-silane to its structure-inducing activities. The retention of alpha-helical conformation upon incorporation of 3F-BLG complexes into TEOS sol-gels directly supports use of 3F as a
polymerizable analog of fluoroalcohols for the CPC-MIP.

EXPERIMENTAL

Materials

(3,3,3-Trifluoropropyl)trimethoxysilane (3F, > 95% purity, MW = 218.3, d = 1.14 g ml\(^{-1}\)), tetraethylorthosilicate (TEOS, > 99% purity, MW = 208.3, d = 0.94 g ml\(^{-1}\)), and n-propyltrimethoxysilane (nPM, > 95% purity, MW = 164.3, d = 0.94 g ml\(^{-1}\)) were purchased from Gelest, Inc. (Morrisville, PA). Analytical grade 2,2,2-trifluoroethanol (TFE, 99% purity, MW = 100, d = 1.393 g ml\(^{-1}\)) and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, > 98% purity, MW = 168, d = 1.622 g ml\(^{-1}\)) were purchased from Fisher Scientific (Hanover Park, IL). Beta-lactoglobulin from bovine milk (BLG, > 90% purity, MW = 18,400) was obtained from Sigma-Aldrich (St. Louis, MO). The lipophilic probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS, > 95% purity) was purchased from Molecular Probes, Inc. (Eugene, OR).

Sample preparation

Both low pH and low temperature favor silane hydrolysis with minimal condensation.\(^{23}\) The stock solution (1.2 M) of hydrolyzed silane sol was prepared by adding 2.66 ml TEOS, 2.30 ml 3F and 2.11 ml nPM with 20 mM HCl at a volume of 7.34 ml, 7.70 ml and 7.89 ml, respectively. Following the ultra-sonication in an ice bath for 30 minutes, TEOS in the acid was stir-mixed (600 rpm) in the ice bath for approximately five to six hours until uniform viscosity was obtained. Both 3F and nPM hydrolyzed faster than TEOS in HCl, in which the acid mixture of 3F or nPM were vortex-mixed for two to five minutes and then placed at 4°C until the silane become completely miscible.
with water (about 24 hours for 3F and six to eight hours for nPM).

Stock solutions of BLG (5 mg/ml) and 1,8-ANS (0.1 mg/ml) were all prepared by dissolving 50 mg BLG and 20 mg 1,8-ANS in 10 ml and 20 ml 20 mM HCl, respectively. BLG was dissolved by gentle swirling, and the dissolving of 1,8-ANS in the acid was facilitated with an ultrasonication bath (Fisher scientific, Hanover Park, IL) for one hour.

**Trp and 1,8-ANS fluorescence in pre-polymerization complex**

The steady-state fluorescence intensity of Trp and 1,8-ANS were monitored by using a Synergy 4 monochromator fluorometer (BioTek Instruments, Winooski, VT) facilitated with a reader for 96-well plates. The protein intrinsic fluorescence was monitored by setting the excitation wavelength at 290 nm in order to exclude minor emissions from tyrosine residues. The emission of Trp was monitored at 330 nm. The excitation wavelength for 1,8-ANS was set at 380 nm, and the emission was collected at 470 nm. All steady-state fluorescence intensity was recorded at 20°C following equilibration in the dark for 30 minutes at room temperature.

To measure the fluorescence of 1,8-ANS binding with BLG in the presence of a cosolvent (hydrolyzed silane sols or the fluoro-alcohols), 74 μl BLG (5 mg/ml, in 20 mM HCl) was mixed with 300 μl 1,8-ANS (0.1 mg/ml in 20 mM HCl) and a 626 μl of a hydrolyzed silane sol or a fluoro-alcohol that was diluted by 20 mM HCl. The concentration range of the silanes and the alcohols in the sample mixtures were 0.1 M - 1.2 M and 0.1 M - 3.5 M, respectively. The sample mixtures for monitoring Trp fluorescence were prepared by the same way as the above but without the addition of the probe 1,8-ANS. The fluorescence of protein-free blank that was around 300 RFU (relative fluorescence unit) was subtracted from that of each BLG-containing samples
(about 100- to 300-fold higher fluorescence than the blank). The probe emission data were normalized as the ratios of sample fluorescence to that of cosolvent-free control (F/F₀).

Due to the spatial proximity of Trp residues and 1,8-ANS binding sites on BLG, a non-radiative fluorescent energy transfer occurs between the two chromophores. Therefore, the fluorescence ratio of the two fluorophores (I_{AN}/I_{Trp-ANS}) was used to demonstrate the FRET efficiency, which was also normalized as the ratio of sample FRET to that of cosolvent-free control (I/I₀). The normalized fluorescence or FRET was plotted versus the concentration of the fluoro-alcohol or the silane. Each fluorescence data point is the average of at least four independent replicates. The standard deviations of the data were within ± 5%, and the error bars are omitted for clarity.

**1,8-ANS fluorescence in BLG-entrapped TEOS gel**

The hydrolyzed TEOS sol was prepared by mixing 4.5 ml TEOS, 1.4 ml H₂O (ddH₂O), 0.1 ml of 0.1 M HCl and ultra-sonicating (Fisher scientific, Pittsburg, IL) in an ice bath for 30 minutes, followed by magnetic stir-mixing in an ice bath for 5 - 6 hours until uniform viscosity was obtained. Before use, the TEOS sol (3.4 M, near pH 2) was stored in a freezer at -20°C for at least seven days to promote full hydrolysis.

3F or nPM-modified TEOS condensation mixture was prepared by first mixing BLG (5 mg/ml, in 20 mM HCl) with hydrolyzed 3F / nPM (1.2 M, in 20 mM HCl), which was then added to hydrolyzed TEOS sol and condensation initiated by adding hepes buffer (0.01 M pH 8.0) at various volume ratios to yield BLG concentration at 0.37 mg/ml and the concentrations of 3F or nPM ranging from 0.04 M to 0.24 M (the total silane was 0.85 M in each sample).
To evaluate the influence of 3F on the protein structure in TEOS gel, HFIP and TFE were entrapped in pure TEOS gels by mixing the fluoro-alcohols in the condensation mixture. To prepare 1 ml the alcohol-containing condensation mixture, 250 μl hydrolyzed TEOS sol was stir-mixed with 74 μl BLG (5 mg/ml) and various volume ratios of the fluoro-alcohol with hepes buffer (0.01 M pH 8.0) to yield HFIP / TFE concentrations ranging from 0.2 M to 1.5 M. The pH values of all condensation mixtures were around pH 4.5.

A 200 μl aliquot of each condensation mixture was pipetted into the well of a 96-well plate, covered with a lid and sealed with Parafilm for controlled polycondensation at 4ºC. The gel formation was defined as when flow was no longer visibly observed in tilted wells. The 3F / TEOS gel and the gels entrapped with the fluoro-alcohols polycondensed in approximately 24 hours, while nPM / TEOS took almost 48 hours to form the gel. The fluorescence of 1,8-ANS binding with BLG in the gels was monitored by adding 24 μl 1,8-ANS (0.25 mg/ml, pH 4.8 hepes buffer) on each gel, and the probe emission at 470 nm was collected after 1 hour probe diffusion at room temperature. All fluorescence data was normalized as the ratio of sample fluorescence to that of the silane or alcohol-free control (F/F₀). Each sample is the average of four independent replicates.

**Circular dichroism spectroscopy**

BLG stock (5 mg/ml) was diluted to 0.1 mg/ml by 20 mM HCl, and 2.5 ml of the diluted protein was pipetted into a quartz cuvette. The fluoro-alcohols or the hydrolyzed silane sols (1.2 M, in 20 mM HCl) were added by magnetic stir-mixing to each cuvette at volumes ranging from 80 μl to 300 μl at room temperature. The circular dichroism (CD)
spectra between 190 to 270 nm were then monitored by an Aviv 410 Circular Dichroism Spectrophotometer (Aviv, Japan). Each sample was scanned in triplicate at a rate of 2 nm a second.

RESULTS AND DISCUSSION

3F-induced structural transitions in BLG

To investigate the influence of the 3F monomer on the structure of the template in pre-polymerization complex, 3F was hydrolyzed at approximately pH 2 and allowed to interact with BLG. Such a low pH favors the maximal hydrolysis and the minimum condensation of the trifluoro-silane, and the reaction condition allows 3F to interact with the native structure of BLG, as the protein dimer (at neutral pH) dissociates into monomer but retains a native conformation at acidic environment (pH < 3.5).24,25

Figure 4.1 shows the dependence of the protein intrinsic fluorescence intensity on the concentrations of silane / alcohol in 20 mM HCl. The intrinsic fluorescence of protein is generally due to the emission of aromatic amino acid side chains, particularly tyrosine and tryptophan.26 When excitation wavelength was set at 290 nm, the intrinsic fluorescence is dominated by tryptophan emission, excluding the minor emission from tyrosine.25 As seen from the result, hydrolyzed TEOS did not show any resolved fluorescence changes at the investigated concentrations (< 1.2 M). TFE reduced Trp emission only at concentrations beyond 2 M, whereas nPM was active at that > 0.8 M. In contrast, both hydrolyzed 3F and HFIP led to dramatic decreasing of the protein intrinsic fluorescence, suggesting the substantial BLG structure transitions induced by these compounds that lead to increased solvent exposure of tryptophan residues and the altered
dielectric around the tryptophan residues. At concentration < 1.2 M, 3F-induced the reduction of Trp emission is up to two-fold greater than HFIP, indicating the stronger perturbation of the compact structure of BLG than the benchmark fluoro-alcohol. The order for the cosolvents to induce Trp fluorescence is 3F > HFIP > nPM >> TFE / TEOS.

**Figure 4.1** The dependence of the intrinsic Trp fluorescence intensity of BLG on concentration of the fluoro-alcohols or the hydrolyzed silanes as indicated in 20 mM HCl. BLG concentration in each sample was 0.37 mg/ml. The standard deviations were within ± 5%, and the error bars are omitted for clarity (n = 4).

To further detect 3F-induced BLG transition state(s) and the protein domains involved in the process, the dependence of 1,8-ANS fluorescence intensity on the concentrations of 3F was monitored in 20 mM HCl. Figure 4.2 depicts the steady-state fluorescence intensity of 0.03 mg/ml 1,8-ANS binding with 0.37 mg/ml BLG in 20 mM HCl in the presence of the silanes / alcohols. Due to the enhanced quantum yield of 1,8-ANS fluorescence in a low dielectric environment, the fluorescence increased in the presence of the silanes / alcohols that induced conformational changes in BLG. At 0.2 M, 3F-induced ANS fluorescence reached a maximum, and then sharply dropped at higher
concentrations. Following the same trend as 3F, HFIP and hydrolyzed nPM each led to the formation of a 1,8-ANS fluorescence maxima. However the most distinct differences between the three cosolvents-induced conformational shifts are follows: (1) 3F induces a fluorescence maximum at a concentration about one-third that of HFIP and one-fourth of nPM (0.6 M HFIP and 0.8 M nPM), (2) the fluorescence rapidly drops after the maximum upon increasing of 3F concentration, whereas a broader transition was observed for either HFIP and nPM. In contrast, elevated concentrations of hydrophilic TEOS had no resolved influence on 1,8-ANS fluorescence emission, and TFE slightly quenched the probe fluorescence at concentration > 2 M.

![Figure 4.2](image)

**Figure 4.2** The fluorescence intensity of 0.03 mg/ml 1,8-ANS as the function of the concentration of fluoro-alcohols (HFIP and TFE) or hydrolyzed silanes (3F, nPM and TEOS) in presence of 0.37 mg/ml BLG in 20 mM HCl. The standard deviations were within ± 5%, and the error bars are omitted for clarity (n = 4).

The fluorescence probe 1,8-ANS itself has almost no fluorescence in water,
whereas it has a high affinity with the hydrophobic pockets of protein that lead to greatly enhanced fluorescence (quantum yields approximately 0.7).\textsuperscript{27,28} Protein unfolding further increases the 1,8-ANS fluorescence due to the exposure of hydrophobic binding sites, while the formation of a molten globule intermediate leads to the maximum 1,8-ANS binding with protein. Therefore, 1,8-ANS has also been used widely for detection of molten globule intermediate.\textsuperscript{16,17,19-22} The 1,8-ANS fluorescence data in Figure 4.2 clearly show hydrolyzed 3F / nPM, the water soluble yet hydrophobic silanes, and hydrophobic alcohol HFIP, each inducing a concentration-dependent fluorescence maximum that may be attributed to an equilibrium state such as a molten globule.\textsuperscript{29,30} The trifluoro-silane 3F exhibited stronger structure-inducing activity than the benchmark fluoro-alcohol and the non-fluorinated silane nPM by being active at lower concentration and within the a narrower window.

Thus, our data from both intrinsic and 1,8-ANS fluorescence assays indicate changes in BLG conformation induced by 3F, nPM and HFIP. These shifts can be further evaluated in terms of the fluorescence resonance energy transfer between Trp and protein-bound 1,8-ANS as shown in Figure 4.3. The insert fluorescence spectra clearly shows the dose-dependent decreasing of Trp fluorescence and increasing of the probe emission with the addition of 1,8-ANS in protein solution. Thus, the fluorescence intensity ratio of the probe to that of Trp ($I_{\text{ANS}} / I_{\text{Trp-ANS}}$) is used to demonstrate the FRET efficiency. Our results show that the energy transfer efficiency is strongly dependent on the cosolvent concentration, with FRET maxima occurring at 0.2 M 3F, 0.6 M HFIP and 0.8 M nPM. As compared to the FRET efficiency in cosolvent-free control, that induced by 3F, nPM, and HFIP increased 8.6, 4.4, and 3.3 folds, respectively. In contrast, no significant FRET
occurred in the presence of TEOS sol and TFE. Taken together, our intrinsic fluorescence data, the probe fluorescence data, and the FRET data are consistent with each other, all the results clearly point out the stronger structure influence activity of 3F than both nPM and HFIP, while the sol-gel matrix monomer TEOS and the trifluoro-alcohol TFE have no significant influence on BLG conformation.

**Figure 4.3** The energy transfer efficiency between 1,8-ANS and BLG Trp residues, presented as the ratio of 1,8-ANS fluorescence to the intrinsic fluorescence of ANS-bound BLG ($I_{\text{ANS}} / I_{\text{Trp-ANS}}$). The probe 1,8-ANS (0.03 mg/ml) was mixed with BLG (0.37 mg/ml) in the presence of indicated fluoro-alcohols or hydrolyzed silane sols in 20 mM HCl. **Insert:** The fluorescence spectra of 1,8-ANS mixing with BLG at the indicated molar ratios of probe/BLG. The probe binding led to the increasing of the emissions at 480 nm but the decreasing of Trp fluorescence at 330 nm. The fluorescence ratio between the two was used to represent the energy transfer between 1,8-ANS and Trp fluorophores.
Figure 4.4 The structure of bovine beta-lactoglobulin (BLG). Left: eight parallel beta-sheets (A to H) form the core hydrophobic calyx, with two tryptophan residues, Trp 19 and Trp 61, residing peripherally (Adopted from Ref. 25). Right: 1,8-ANS interacts with the hydrophobic beta calyx in BLG with its sulphate group in close contact with lysine residues Lys 60 and Lys 69 (Modified from Ref. 32).

BLG is a globular protein (MW 18,400) and is predominantly beta-sheet structured, consisting of one major alpha-helix and a beta-barrel of eight continuous anti-parallel beta-strands. The protein has two Trp residues at amino acid positions 19 and 61. Trp 61 is located in a solvent accessible surface cleft, and Trp 19 is located at the bottom of a central hydrophobic calyx formed by anti-parallel beta-strands (Figure 4.4). Previous studies have revealed that BLG possesses two different binding sites for 1,8-ANS: an external site, in proximity of a hydrophobic patch on the protein surface, and an internal site located in the central hydrophobic calyx of BLG (Figure 4.4). The external site has a nonspecific interaction with 1,8-ANS, whereas at the internal site the negatively charged 1,8-ANS sulphonate group is in close electrostatic mediated contact with two Lys residues. Hence, the fluorescence of 1,8-ANS is primarily attributed to its binding within the BLG hydrophobic calyx where Trp 19 is located. Conformational shifts that bring the BLG-bound 1,8-ANS closer to Trp19 will increase fluorescence yield, as FRET is only effective for acceptor-donor separations on the order of a few nanometers.
Thus, the remarkably increased FRET by 3F reveals that the trifluoro-silane induced significant conformation transitions around the central hydrophobic environment, which is attributed to the loss of the native beta-sheet structures that places Trp19 in closer proximity to the 1,8-ANS binding site during the formation of the molten globule-like intermediate. This transition from beta-sheet to alpha-helix is further supported by the circular dichroism data in Figure 4.5, where distinct minima at 208 and 222 associated with alpha-helices are clearly observed at 3F concentrations between 0.2 and 0.3 M.

![Figure 4.5](image)

**Figure 4.5** Circular dichroism spectrum of 3F-induced alpha-helical structure (minima at 208 and 222 nm) over the silane concentration range as indicated. From top to bottom, the spectra correspond to 3F concentrations of 0, 0.04, 0.05, 0.09 and 0.13 M mixed with 0.1mg/mL BLG in 20 mM HCl.

**Conformation induction in fluoro- and alkyl-sol-gels**

The previous data have demonstrated the strong activity of hydrolyzed 3F monomers on BLG conformation in 0.01 M 20 mM HCl. For molecular imprinting, the
conformational shifts induced in the pre-polymerization complex must be preserved upon cross-linking. Figure 4.6 shows the emission intensity of 1,8-ANS in TEOS gel modified with 3F / nPM or entrapped with HFIP / TFE. From the results, polymerized silane and HFIP in the gels all exhibited similar influences on the probe fluorescence as in the solution: each of them led to a maximum emission of 1,8-ANS, with 3F / nPM being effective at a concentration less than half of HFIP (0.43 M HFIP vs. 0.17 M 3F or nPM). Additionally, the maximum 1,8-ANS emission intensity induced by the copolymerized 3F was even slightly higher than that by gel-entrapped HFIP, indicating the stronger structure-inducing activity of 3F in TEOS gel. Interestingly, TFE began to increase the probe emissions at higher concentrations (> 0.9 M), suggesting the importance of

![Figure 4.6](image-url)

**Figure 4.6** The fluorescence intensity of 1,8-ANS binding with BLG in TEOS gel either entrapped with the fluoro-alcohols (HFIP and TFE) or copolymerized with 3F or nPM. Each gel (200 μl) contained 0.37 mg/ml BLG and was diffused with 24 μl 0.25 mg/ml 1,8-ANS (in 0.01 M pH 4.8 hepes buffer). The standard deviations were within ± 5%, and the error bars are omitted for clarity (n = 4).
confined environments on solvent- / silane-induced conformational shifts. The restricted environment in the gels may also account for the four-fold enhanced 1,8-ANS fluorescence observed in the gels (Figure 4.6) as compared to free solution (Figure 4.2).

The spatial distribution of protein-bound 3F that supports an alpha-helical conformation is readily preserved by adding TEOS to the pre-equilibration mixture followed by raising the pH or temperature to induce polycondensation of 3F and TEOS around the template protein. Our study shows that 3F-induced BLG structure transitions can be retained when 3F is copolymerized with TEOS in the gel, as inferred from the data that 0.17 M polycondensed 3F-induced 1,8-ANS binding with BLG producing a fluorescence peak slightly higher than that induced by 0.43 M HFIP (Figure 4.6). This data fully supports the premise that not only can a hydrolyzed fluoro-silane induce an alpha-helical structure in a pre-complex in solution but also the biological activity associated with this structure is preserved upon gelation. From this standpoint, we view 3F as superior to HFIP in inducing structural transitions, with dual functionality as a functional monomer for molecular imprinting.

Kanjila et al. correlated 1,8-ANS fluorescence with the compressibility changes of BLG and demonstrated the conformational transition from beta-sheet to alpha-helix in the presence of 20% (v/v) HFIP. Circular dichroism data from Hirota et al. also revealed the cooperative formation of alpha-helices by 6% (v/v) HFIP. Above the volume ratio of 8%, the rigid environments around the aromatic amino acids of the native state had been completely lost. Our circular dichroism data and fluorescence data of 3F are in agreement with these studies of HFIP inducing the beta-sheet to alpha-helix transition in BLG.
Modes of structure inducing activity

BLG immobilization in TEOS appears to prime the protein for structural changes as evidenced by the enhanced activity of TFE on gel-entrapped protein (Figure 4.6) compared to almost no activity by TFE on BLG in solution (Figure 4.1 - 4.2). The multiple constituent halogen groups in HFIP point to the important role of fluorine in inducing and stabilizing helical structure in protein. However, the significantly greater structure-inducing activity of the trifluoro-silane 3F over the trifluoro-alcohol TFE implicates a contributing role of alkylsilanol(s) in the conformation transitions.

Hirota et al. compared the effectiveness of alkanols with variable hydrocarbon groups and demonstrated that the bulkiness of hydrocarbon groups was proportional to the effectiveness of alcohol-induced alpha-helix formation. Our results that nPM, as the propyl substitution of TEOS, has stronger activity than nPM in inducing BLG conformational transitions suggest the positive contribution of the alkyl groups for the structure-inducing activity. Moreover, 3F is a even stronger conformation inducer than nPM, revealing that the importance of the trifluoro groups. Hydrolyzed TEOS is composed of four silanol groups (Si-(OH)₄). The substitution of bulky groups such as propyl (in nPM) or trifluoro-propyl (in 3F) (Figure 4.7) disrupts the symmetric structure of TEOS, which could lead to enhanced electrostatic and hydrophobic interactions with protein. Moreover, the trifluoro groups may promote 3F-protein interaction by forming H-bonds with hydrogen donors on protein residues, which consequently disrupts the intramolecular H-bonds formed between distant residues and favors those formed by proximal amino acids on polypeptide chain, leading to increased propensity of alpha-helices. Retention of activity once polymerized in the TEOS-gels validates use of
the trifluoro-silane as functional monomers for constructing catalytic protein conformational imprint proposed here.

\[ \text{(a) (3,3,3-trifluoropropyl) trimethoxysilane (3F)} \quad \text{(b) n-propyltrimethoxysilane (nPM)} \quad \text{(c) tetraethylorthosilicate (TEOS)} \]

![Chemical Structures](image)

**Figure 4.7** The chemical structures of the silanes TEOS, nPM and TEOS, and silanols hydrolyzed from each silane by acid catalysis.

**CONCLUSIONS**

We report here a first investigation of a trifluoro-silane monomer that is capable of inducing protein conformational changes similar to those observed for protein-fluoro-alcohol interactions. The hydrophobic 3F was investigated as a structural analogue of the fluoro-alcohol TFE, but it exhibited much stronger activities than TFE on inducing a molten globule-like intermediate accompanied by the formation of alpha-helix in BLG. Moreover, 3F exhibited greater influence on BLG structural transitions than the benchmark alpha-helix-inducing fluoro-alcohol HFIP. The strong structure-inducing activity of 3F was retained upon the polycondensation in TEOS matrix. In contrast, TEOS had no resolved influence on conformation of BLG, validating its application as an inert matrix for the protein conformational imprinting. By using nPM as the control, we
demonstrated that the alkyl chain and the trifluoro groups contribute cooperatively to the strong structure transition activity of 3F.

While only one or two ANS binding sites are generally discussed for the template protein BLG, there may be multiple sites where fluoro-silanes interact with the protein. For MIP technology, these results represent a major breakthrough in developing functional monomers that interact with template through hydrophobic (fluoro and alkyl) interactions. The strong activity on protein conformation supports the use of the trifluoro-silane as a functional monomer for catalytic protein conformational imprinting.

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CHAPTER 5
ENZYME ACTIVITY AND CONFORMATIONAL CHANGES
OF BOVINE CARBONIC ANHYDRASE IN
TRIFLUORO-SILANE MODIFIED
SOL-GELS*

Abstract: Protein conformational imprinting is a variation of molecularly imprinted polymers (MIPs) in which functional monomers are selected to induce or stabilize a desired secondary structure in the template protein. Acid hydrolyzed 3,3,3-trifluoropropylmethoxysilane (3F) is employed here as an alpha-helix-inducing functional monomer that retains activity once immobilized in tetraethylorthosilicate (TEOS) gels. The extent to which 3F in TEOS affects an immobilized protein structure and the enzyme activity was tested using bovine carbonic anhydrase (BCA) as a template protein. Hydrolyzed 3F-induced 1,8-ANS fluorescence changes with time and pH. In pH 2.5 hepes buffer two fluorescence peaks corresponding to MG-transitions evolved over 24 hours, while in pH 4 hepes buffer a single MG-transition peak was observed. The 3F-BCA interaction in the pre-polymerization complex was retained upon polymerization in TEOS gel, where single sharp peaks corresponding to MG-transitions were observed at 0.17 M 3F. Relative to BCA entrapped in pure TEOS, the enzymatic activity (I/I₀) in 3F-TEOS was maintained at around 84% between 0.04 M and 0.11 M 3F, then steadily decreased to 30% at 0.2 M 3F and fell below 10% at 0.24 M 3F within a 24 hour assay period. The 3F dependent loss of enzymatic activity for gel-entrapped BCA illustrates the strong conformational activity of the trifluoro-silane condensed in a silicate matrix. The conformation inducing activity of 3F is demonstrated to be greater than the benchmark

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fluoro-alcohol, 1,1,1,3,3-hexafluoro-2-propanol (HFIP), which induced a MG peak for BCA encapsulated in TEOS at 0.41 M HFIP. By combining enzyme activity studies with fluorescent spectroscopy analysis of protein structure we demonstrate that 3F is a biologically active functional monomer suitable for inducing defined structural transitions and conformations in target proteins.

**INTRODUCTION**

The proper folding of protein structures is of vital importance to their biological functions. The well-defined three-dimensional structure of proteins not only determines their long term stability and specific interaction with receptors and ligands, but also links essentially with numerous biological processes, ranging from molecular recognition and transportation to cell growth and regulation. In many cases, misfolded proteins are associated with a wide variety of pathological conditions, including some of the currently most perplexing medical problems such as Alzheimer’s disease, Parkinson’s disease and the prion encephalopathies. Despite the diverse clinical presentations, the molecular basis of these neurodegenerative disorders all involve destabilization of alpha-helices and simultaneous formation of beta-sheets, which lead to “multimerization” of misfolded proteins into insoluble extracellular aggregates and / or intracellular inclusions such as cross-beta sheet amyloid fibrils. Thus, stabilization of alpha-helix and / or disruption of beta-sheet formation to inhibit the subsequent self-association to form different pathological macrostructures are among the most important therapeutic strategies for addressing these causative agents of conformational diseases.

Molecular imprinting is the synthesis of highly cross-linked polymers that contain template molecule-induced binding cavities complementary to both the spatial
configuration and the functionality of target molecules. The complementarity can be improved through the selection of functional monomers that exhibit high affinity for specific chemical moieties or regions on the template molecule. Such molecularly imprinted polymers (MIPs) keep a memory of the template and can be customized to recognize various target molecules with antibody-mimic affinities and selectivity. Although MIP technology was developed only half a century ago, many imprinted polymers have been successfully applied in the fields of solid phase extraction, enantiomer separation, sensor fabrication, drug discovery and controlled drug release.\textsuperscript{7-17} In the last 20 years, chemically synthesized imprints for recognition of biological macromolecules, especially proteins, have been intensively studied.\textsuperscript{18}

Here, a protein conformational imprinting technology is proposed for inducing structure transitions in template proteins, particularly the formation and / or stabilization of alpha-helices. To achieve this aim, the selection of functional monomers is of primary importance. In the previous chapter, the efficacy of (3,3,3-trifluoropropyl)-trimethoxysilane (3F) in inducing conformational transitions from beta-sheet to alpha-helix in bovine beta-lactoglobulin in a tetraethylorthosilicate (TEOS) matrix has been demonstrated. The fluoro-silane 3F is employed as a polymerizable analogue to 2,2,2-trifluoroethanol (TFE), which is well known for inducing alpha-helices in many proteins.\textsuperscript{19} To further validate conformational imprinting, bovine carbonic anhydrase (BCA) is employed as the template protein to explore the effects of 3F on BCA enzymatic activity. In addition, the steady-state fluorescence intensity of the probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) was monitored as a function of 3F concentration. As 1,8-ANS fluorescence is greatly enhanced upon binding with
hydrophobic pockets of proteins, it is widely used to interrogate protein structural transitions.\textsuperscript{20-24} The combined enzymatic and fluorescence studies reveal a modest decrease in enzymatic activity that corresponds to the onset of a molten globule (MG) type transition in BCA. As the 3F concentration in the gel is increased, the enzymatic activity of BCA further decreases, corresponding to the completion of the transition. From these studies, the effectiveness of 3F as a functional monomer for inducing structural transitions in protein is clearly demonstrated. The protective role of TEOS encapsulation on enzyme activity is probed using urea as denaturant that is either present during encapsulation or diffused into the pre-formed BCA-TEOS gel.

\textbf{EXPERIMENTAL}

\textbf{Materials and sample preparation}

(3,3,3-trifluoropropyl)trimethoxysilane (3F, \(> 95\%\) purity, MW = 218.3, \(d = 1.14\) g ml\(^{-1}\)), \(n\)-propyltrimethoxysilane (nPM, \(> 95\%\) purity, MW = 164.3, \(d = 0.94\) g ml\(^{-1}\)) and 3- tetraethylorthosilicate (TEOS, \(> 99\%\) purity, MW = 208.3, \(d = 0.94\) g ml\(^{-1}\)) were purchased from Gelest, Inc. (Morrisville, PA). Analytical grade 2,2,2-trifluoroethanol (TFE, \(99\%\) purity, MW = 100, \(d = 1.393\) g ml\(^{-1}\)), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, \(> 98\%\) purity, MW = 168, \(d = 1.622\) g ml\(^{-1}\)) were all purchased from Fisher Scientific (Hanover Park, IL). Bovine carbonic anhydrase (BCA, \(> 90\%\) purity, MW = 30,000) and \(p\)-nitrophenyl acetate (pNPAC, \(> 98\%\) purity) was purchased from Sigma-Aldrich (St.Louis, MO). The fluorescence probe 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS, \(> 95\%\) purity) was obtained from Molecular Probes, Inc. (Eugene, OR). Hepes buffer (pH 7, 1 M) and urea (\(> 99\%\) purity) were purchased from Fisher Scientific (Hanover Park, IL).
The probe solutions (0.5 mg/ml) were prepared at two pH values by dissolving 10 mg of 1,8-ANS in 20 ml 0.01 M pH 2.5 or pH 4 hepes buffer and ultrasonicating (Fisher Scientific, Hanover Park, IL) for one hour. BCA solutions were prepared at three pH values: pH 7.5 BCA (0.5 mg/ml) was prepared by dissolving 10 mg BCA in 20 ml pH 7.5 hepes buffer (0.01 M); pH 2.5 and pH 8 BCA (3 mg/ml) were prepared by dissolving 15 mg BCA in 5 ml 0.01 M pH 2.5 and pH 8 hepes buffer, respectively. The protein in each solution was dissolved by gentle swirling. pNPAc solution (50 mM) was prepared by dissolving 181 mg pNPAc in 20 ml acetonitrile and was stored at -20 °C. Urea stock solution (5 M) was prepared by dissolving 6 g urea in 20 ml 0.01 M pH 7.5 hepes buffer.

**BCA enzyme activity in solution**

BCA activity via pNPAc esterase assay was modified based on the method by Rozema and Gellman,\(^{25}\) in which BCA acts as an esterase catalyzing the hydrolysis of pNPAc, the ratio of which represents the BCA activity. To measure BCA enzyme activity in solution, a 200 μl aliquot of a protein dilution (6.25 μg/ml, in 0.01 M pH 7.5 hepes buffer) was pipetted into each well of a 96-well plate. Immediately after the addition of 50 μl pNPAc (25 mM), the hydrolysis of the substrate was monitored spectrophotometrically (Thermolabsystems, VA) by measuring the absorbance at 420 nm over 60 seconds. Each sample is the average of four independent measurements.

**BCA activity in TEOS gels with urea treatments**

The hydrolyzed TEOS sol was prepared by stir-mixing (600 - 800 rpm) 4.5 ml TEOS, 1.4 ml ddH₂O, 0.1 ml of 0.1 M HCl and ultra-sonicating (Fisher Scientific, IL) in an ice bath for 30 minutes, followed by magnetic stir-mixing (600 rpm) for approximately six hours until uniform viscosity was obtained. Before use, the TEOS sol
was stored in a -20°C freezer at least for seven days to promote full hydrolysis.

To test the stabilizing effect of TEOS against urea denaturation of encapsulated BCA, two gels were prepared: BCA-TEOS gels into which urea was diffused, and urea pre-mixed BCA-TEOS gels. Urea-free controls and urea-diffused gels were prepared by stir-mixing (800 rpm) 250 μl hydrolyzed TEOS sol with 700 μl hepes buffer (0.01 M pH 8.0) and 50 μl BCA (0.5 mg/ml pH 7.5). Urea-premixed gels were prepared by adding urea (5 M pH 7.5) to a BCA-TEOS pre-condensation mixture at appropriate volume ratios to yield urea concentrations ranging from 0.1 M to 3.5 M. NaOH (1N, 5 - 7 μl) was stirred mixed (800 rpm) to initiate condensation by raising the pH to approximately 6.2.

A 50 μl aliquot of each condensation mixture was pipetted into the wells of a 96-well plate (Corning Inc., NY). The gel formation was defined as when flow was no longer visibly observed in the tilted wells. At about pH 6.2 gels formed in each well within 10 minutes at room temperature. To attain a uniform gel pH, pure TEOS gel was incubated with 0.01 M pH 7.5 hepes buffer (500 - 600 μl ) at 4 °C for one hour, while urea dilutions (0.1 M to 3.5 M, in pH 7.5 hepes buffer) were applied for urea-premixed gels. The urea-diffused gel was prepared by incubating 200 μl urea dilutions (0.1 M to 0.3 M) with 50 μl pure TEOS gels at 4°C for two hours. To demonstrate the activity recovery of denatured enzyme in gel, the denaturant in a urea-premixed gel was diluted out by incubating with 200 μl hepes buffer (0.01 M pH 7.5) for one hour.

Before the assay, all plates prepared above were placed upside down to drain the gels for two - five minutes. The absorbance was then monitored after the addition of 25 μl 50 mM pNPAc and 175 μl appropriate buffer or urea dilution (in pH 7.5 hepes buffer) in each well. All enzyme data in gel were recorded at a time interval of 10 seconds after the
initial 30-minute incubation. Each data point is the average of eight independent measurements and is normalized by using the ratio of sample absorbance to that of urea-free control (I/I_0).

**BCA activity in 3F-modified TEOS gel**

Acid catalyzed hydrolysis of 3F / nPM sol (1.2 M) was prepared by mixing 2.3 ml 3F and 2.11 ml nPM with 0.01 M pH 2.5 hepes buffer at volumes of 7.7 ml and 7.89 ml, respectively. The mixtures were stored in 4°C until the hydrolyzed silane solutions were completely miscible with water (about 24 hours for 3F and six to eight hours for nPM). To encapsulate protein in 3F- or nPM-doped TEOS gel, BCA (0.5 mg/ml, pH 2.5) was mixed with hydrolyzed 3F / nPM (1.2 M, in pH 2.5 hepes buffer), which was then added to hydrolyzed TEOS sol and hepes buffer (0.01M pH 8.0) at various volume ratios to yield BCA concentration as 0.025 mg/ml and that of 3F or nPM ranging from 0.04 M to 0.24 M, keeping the total silane as 0.85 M in each mixture. NaOH (1N, 5 - 7 μl) was stir-mixed (800 rpm) with each sample mixture to raise the pH to approximately 6.2 to initiate the condensation. Following the gel formation after 10 - 15 minutes, each gel was rinsed by hepes buffer (0.01 M pH 7.5) and the absorbance at 420 nm was monitored the same way as described above. The data were normalized as the ratios of the sample absorbance to that of pure TEOS gel (I/I_0).

**Fluorescence spectroscopy**

The steady-state fluorescence intensities were recorded on a Synergy 4 monochromator fluorimeter (BioTek Instruments, Winooski, VT) equipped with a 96-well plate reader. The fluorescence of 1,8-ANS was monitored by setting the excitation wavelength at 380 nm and the emission wavelength at 470 nm.
Protein and probe fluorescence in buffer. The 1,8-ANS fluorescence shifts induced by hydrolyzed silanes held in solution at pH 2.5 were monitored by mixing 200 μl BCA (3 mg/ml, pH 2.5) with 300 μl 1,8-ANS (0.1 mg/ml, pH 2.5) and 500 μl of a cosolvent (1.2 M hydrolyzed 3F/ nPM or HFIP / TFE) and hepes buffer (0.01 M pH 2.5) at various ratios to yield cosolvent concentrations ranging from 0.1 M to 1.5 M. The sample mixtures for fluorescence assays at pH 4 were prepared by using 0.5 N NaOH to adjust the pH 2.5 mixture to pH 4 (2 - 5 μl). A 200 μl aliquot of each sample mixture was pipetted into the wells of a 96-well plate (Corning black polypropylene, Corning, NY). The emissions of the fluorophores were first recorded at an interval of about one or two hours for up to five hours at 20°C, and a final assay was monitored after each sample was stored at 4 °C for approximately 24 hours. Before each assay, the plates were equilibrated at room temperature for at least 30 minutes after sample mixing or storage at 4°C.

Protein and probe fluorescence in gel. TEOS condensation mixture doped with 3F or nPM was prepared by the same protocol as that for 3F / nPM doped gels for enzyme activity assay except that 3 mg/ml BCA (pH 2.5) were used to yield protein concentration of 0.6 mg/ml in the condensation mixture. The concentration of 3F / nPM ranged from 0.04 M to 0.24 M (total silane = 0.85 M). Additionally, fluoro-alcohol containing condensation mixtures were prepared by stir-mixing (600 rpm) 250 μl hydrolyzed TEOS sol with 200 μl BCA (3 mg/ml, pH 2.5) and 550 μl the mixture of HFIP / TFE and hepes buffer (0.01 M pH 8.0) at various volume ratios to yield the fluoro-alcohol concentration ranging from 0.2 M - 1.5 M. The final pH of all condensation mixtures was around pH 4.

A 200 μl aliquot of each condensation mixture was pipetted into the wells of a 96-well plate, covered with a lid and sealed with Parafilm for controlled
polycondensation at 4°C. While nPM modified mixture took approximately 48 hours to form the gel, 3F or fluoro-alcohol-doped mixtures polycondensed in about 24 hours. The fluorescence of 1,8-ANS was then measured by adding 24 μl 1,8-ANS (0.5 mg/ml, pH 4) on each gel and the emission at 470 nm was collected after allowing the probe to diffuse into the gel for one hour at room temperature. All fluorescence data were normalized by using the ratio of the sample gel fluorescence to that of cosolvent-free control or pure TEOS gel (F/F₀). Each sample is the average of four independent measurements.

RESULTS AND DISCUSSION

BCA denaturation and renaturation in TEOS gel

An important prerequisite for protein conformational MIPs is that the polymeric matrix allows template proteins to change their structures freely. In this study, the structure flexibility of BCA in TEOS gel was demonstrated by monitoring the changes of BCA enzyme activities in the gels over a range of urea concentrations at pH 7.5. As shown from the results in Figure 5.1, urea led to a prompt reduction of the enzyme activities when either pre-mixed or diffused into TEOS gels at a concentration < 3 M. After rinsing the urea-premixed gels with hepes buffer (pH 7.5) for one hour, the loss in enzyme activity was recovered almost completely in the gels with urea ≤ 2M, while enzyme activities in gels with urea > 2M were only partially recovered. The reversible denaturation induced by urea (≤ 2 M) in TEOS gel verifies that the TEOS gels prepared in this study provided sufficient spatial freedom for BCA to change structure when encapsulated within the TEOS matrix. The inability to fully recover enzyme activity for the gels with urea > 2M may be due to residual urea in the polymer as well as possible irreversible conformational changes / denaturation at these higher urea concentrations.
Figure 5.1 BCA enzyme activity in hepes buffer (0.01 M pH 7.5) or in TEOS silica gel (pH 7.5) with different urea treatments: × BCA in buffer; ● After the gel polymerization, urea was diffused in the gel to interact with BCA; ▲ Before the polymerization, urea was pre-mixed with BCA in the condensation mixture; ◆ Urea-premixed gel was rinsed with 0.01 M pH7.5 hepes buffer for one hour. BCA concentrations in solution and in gel were 5 μg/ml and 25 μg/ml, respectively. The error bars represent the standard deviations for the data (n = 8).

Figure 5.1 also shows that BCA exposed to < 3 M urea in bulk solution (0.01 M pH 7.5 hepes buffer) exhibited greater activity loss than for TEOS encapsulated BCA. For 3.5 M urea pre-mixed with BCA in the gel the BCA activity was zero compared to about 20% activity for BCA exposed to 3.5 M urea in the bulk. This behavior is attributed to gel porosity leading to a hindered elution rate of urea from the porous gels. This same effect results in a diminished influence of urea diffused into the BCA-TEOS gel over all investigated urea concentrations. Thus, there is a tradeoff between favorable stabilization of BCA confined in TEOS and unfavorable confinement of the denaturant in the porous TEOS matrix. In addition to stabilizing the encapsulated BCA structure and restricting
urea diffusion into the gel, the TEOS may act as a urea sorbent surface, which saturates near 3 M urea, leaving “free” urea able to interact with and denature the encapsulated enzyme. When comparing the trends in Figure 5.1, it is important to note that the enzyme substrate, pNPAc, has a MW three-time greater than urea; thus, any BCA that is completely sequestered from urea will not contribute to $I/I_0$ as pNPAc will not reach these proteins.

**Hydrolyzed 3F-BCA interaction**

From the urea challenges, it is clear that the TEOS gel provides an imprinting / encapsulation matrix permitting sufficient conformational freedom. Before introducing the fluoro-silane 3F as a functional monomer into the gel, the conformation-inducing activity of hydrolyzed 3F in solution with BCA was tested using 1, 8-ANS. This dye is a hydrophobic fluorescence probe exhibiting limited interactions with expanded polypeptide chains but shows high affinity to compact protein hydrophobic pockets.26,27 As compared with native protein structure, a partially folded protein binding with 1,8-ANS always leads to greatly increased probe fluorescence due to largely exposed hydrophobic patches; however the loss of secondary structures due to further unfolding causes decreased 1,8-ANS quantum yield. The fluorescence of 1,8-ANS reaches the maximum when a MG intermediate is formed. Thus the probe has been widely used for demonstration of protein structure changes, particularly the detection of the protein MG intermediate.26,28-33

Figure 5.2 shows that fluorescence intensity of 1,8-ANS induced by hydrolyzed 3F and the non-fluorinated analog nPM changed with time in pH 2.5 0.01 M hepes buffer. Within the first two hours of interaction with BCA, the quantum yield of 1,8-ANS
Figure 5.2 The changes of the fluorescence intensity of 0.03 mg/ml 1,8-ANS as the function of the concentration of hydrolyzed 3F or nPM in 0.01 M pH 2.5 hepes buffer at indicated time of interaction. [BCA] = 0.6 mg/ml in each sample. The error bars present the standard deviation of the data (n = 4).
increased upon the addition of either 3F or nPM, achieving a maximum value near 0.3 M silane. The nPM induced emission, although initially greater than that induced by 3F, steadily decreased and no clear emission peak was discernable at 24 hour. The 3F induced emission also decreased in intensity with time. However, in contrast to nPM two distinct peaks, centered near 0.15 M and 0.4 M 3F, were apparent by 24 hours, suggesting the possible formation of multiple MG-like intermediates as the 3F concentration is steadily increased. The decrease in F/F₀ below unity with increasing silane concentration / time indicates a protein transition from a MG state to more open conformations, eventually adopting a random coil that binds almost no 1,8-ANS (i.e. F/F₀ → 0). The fluoro-alcohols, HFIP and TFE, also exhibited temporal changes in the induced emission profiles as seen in Figure 5.3. A distinct peak that could be attributed to a discrete MG transition was observed for HFIP whereas TFE only led to a slight increase of the emission intensity in a dose-dependent manner.

The 1,8-ANS fluorescence profiles induced by 3F, nPM and HFIP point to significant differences in their respective activities. First, although the maximum 1,8-ANS quantum yield induced by 3F within the first two hours of interaction with BCA was the least compared to HFIP and nPM (HFIP > nPM > 3F), the trifluoro-silane induced the MG state at a concentration similar to nPM (near 0.3 M at 2 hour) but lower than HFIP (near 0.6 M). Second, further interaction of BCA with the three cosolvents led to significantly distinct changes of the probe emission, with 3F leading to the evolution of two emission maxima after 24 hours. As shown in Figure 5.3, HFIP-induced fluorescence slightly decreased with time during the first five hours. However, after 22 hours of interaction with BCA 1,8-ANS emission intensity slightly increased and the peak shifted
Figure 5.3 The fluorescence intensity of 0.03 mg/ml 1,8-ANS a function of the concentration of HFIP or TFE in 0.01 M pH 2.5 hepes buffer and at interaction time intervals as indicated. [BCA] = 0.6 mg/ml in each sample. The error bars represent the standard deviations of the data (n = 4).
from around 0.6 M to 0.75 M. The shift in the 1,8-ANS emission peak to higher HFIP concentrations with time suggests that the protein structure evolves during incubation with HFIP, but not as dramatically as when incubated with nPM or 3F.

The dramatic evolution of fluorescence emission with 3F and nPM may reflect the reactive nature of these silanes. The buffer pH of 2.5 was selected to promote hydrolysis while limiting condensation; however, the evolution of silanols and condensate products was not evaluated other than through visual inspection and turbidity (gelation) analysis. Thus, the ratios of fully hydrolyzed monomers to partial condensation products (e.g. dimmers and trimers) are unknown and could contribute to the evolving activity.

To accelerate the condensation rate, the buffer pH was increased from 2.5 to 4, and 1,8-ANS fluorescence was measured up to 24 hours as presented in Figure 5.4. The 1,8-ANS emission in the presence of nPM or 3F at pH 4 differed dramatically from the corresponding data at pH 2.5. Most notable was the absence of any distinct peak for 3F until a 5-hours incubation time, when a modest increase in fluorescence was observed at 0.4 M 3F. After a 24-hour incubation time this peak increased more than two-fold. Under the same conditions, nPM did not show distinct peaks within the first few hours; however, after 24 hours a small peak appeared centered near 0.2 M nPM with a possible second peak near 0.5 M. At pH 4, the 3F appears to behave as a strict denaturant at early times, whereas at later times it exhibits conformation stabilizing activity as indicated by the evolution of a strong fluorescence maximum. The evolution of two peaks at pH 2.5 (centered at 0.15 and 0.4 M 3F) versus a single peak centered at 0.4 M 3F at pH 4 also indicate that there may be a 3F hydrolysis / condensation product at pH 2.5 that is more active at stabilizing a MG intermediate (first peak at 0.15 M 3F) that is absent at pH 4.
(single peak at 0.4 M 3F).

Figure 5.4 The changes of the fluorescence intensity of 0.03 mg/ml 1,8-ANS as a function of the concentration of hydrolyzed 3F or nPM in 0.01 M pH 4 hepes buffer at indicated time of interaction. [BCA] = 0.6 mg/ml in each sample. The standard deviations of the data were within ± 7%, and the error bars are omitted for clarity (n = 4).
The sol-gel process is outwardly straightforward: a precursor such as silicon alkoxide undergoes hydrolysis and condensation to form a highly polymerized network. The typical sol-gel reactions are as follows:\textsuperscript{34}

**Hydrolysis:**

\[ \text{Si(OR)₄} + n\text{H}_2\text{O} \rightarrow \text{Si(OR)₄-n(OH)}_n + n\text{ROH} \]  

(1)

**Condensation:**

\[ \equiv\text{Si-OH} + \text{HO-Si} \equiv \rightarrow \equiv\text{Si-O-Si} \equiv + \text{H}_2\text{O} \]  

(2)

(water condensation)

and / or

\[ \equiv\text{Si-OR} + \text{HO-Si} \equiv \rightarrow \equiv\text{Si-O-Si} \equiv + \text{ROH} \]  

(3)

(alcohol condensation)

Clearly, multiple reaction pathways can be followed, with the reaction rates and pathways depending strongly on pH, temperature, and presence of nucleophiles. A key aspect of our work is that at pH 2.5 the fluoro-silanes remain in a hydrolyzed state but do not undergo rapid condensation. Salon et al. used \textsuperscript{1}H and \textsuperscript{13}C NMR spectroscopy to show that within four to six hours of reaction initiation, approximately 80\% of a mercapto-silane exists as fully hydrolyzed monomers and about 15\% are dimers; after 14 hours, the dimer percentage is 50\%, and only after 15 hours are any linear polycondensation products observed.\textsuperscript{35,36} These slow hydrolysis rates were attained at 25°C under acidic conditions. Here, 3F was hydrolyzed at pH 2.5 for 24 hours at 4°C prior to mixing with the protein, BCA. Under these conditions, any 3F condensation is likely limited to formation of dimers and short linear chains based on the work by Salon. The greater activity of both 3F and nPM at pH 4 is attributed to the accelerated hydrolysis and condensation kinetics expected at the higher pH. Thus, at longer times the concentration of hydrolyzed monomers decreases while dimer, trimer and linear
polycondensation product concentrations grow. To explore the influence of continued condensation on silane conformation stabilizing activity, we measured 1,8-ANS fluorescence for BCA entrapped in silica gels containing increasing concentrations of 3F or nPM and compared this with BCA in pure TEOS gels into which HFIP or TFE was diffused.

**Polymerized 3F- BCA interaction**

Figure 5.5 depicts the fluorescence intensity of 1,8-ANS diffused into the indicated silane-BCA gels. The results show that polymerized 3F / nPM and HFIP entrapped in a TEOS gel all exhibited a strong influence on probe fluorescence. Each led to a maximum emission of 1,8-ANS on their respective fluorescence transition curves, the quantum yield of which was much greater than that induced by the silanes or fluoro-alcohols in solution (Figure 5.2 - 5.3). As compared to the fluorescence intensity of 1,8-ANS-BCA in pure TEOS gel, the maximum 1,8-ANS emission induced by HFIP was enhanced more than seven-fold, while that by 3F and nPM in the gels increased about 4.5 and 2.6 times, respectively. In pH 2.5 hepes buffer the maximum emission of 1,8-ANS induced by HFIP, 3F and nPM within the beginning were 1.7, 1.3 and 1.4 times higher than the cosolvent-free control (Figure 5.2 - 5.3). In contrast, TFE had almost no influence on 1,8-ANS quantum yield at concentrations below 0.4 M, while above 0.4 M the fluoro-alcohol significantly increased the probe fluorescence, even exceeding that induced by both nPM and 3F at these concentrations. These results suggest that the structure-inducing activities of both the silanes and the fluoro-alcohols were greatly enhanced in TEOS gels. The trifluoro-silane exhibited stronger structure bioactivity than nPM, as inferred from the result that both 3F and nPM induce a MG-like intermediate at
0.17 M, but the maximum quantum yield of 1,8-ANS in 3F-TEOS gel was almost two-fold higher than that in nPM-TEOS gel.

![Graph showing fluorescence intensity of 1,8-ANS after diffusion in TEOS gel modified with 3F or nPM or entrapped with HFIP and TFE. TEOS concentration = 0.85 M – [3F] or [nPM]. Each gel (200 μl) contained 0.6 mg/ml BCA and 120 μl 0.1 mg/ml 1,8-ANS was allowed to diffuse in the gel for 1 hour at 22°C. The standard deviations of the data were within ± 5%, and the error bars are omitted for clarity (n = 4).]

**Figure 5.5** The fluorescence intensity of 1,8-ANS after diffusion in TEOS gel either modified with 3F or nPM or entrapped with HFIP and TFE. TEOS concentration = 0.85 M – [3F] or [nPM]. Each gel (200 μl) contained 0.6 mg/ml BCA and 120 μl 0.1 mg/ml 1,8-ANS was allowed to diffuse in the gel for 1 hour at 22°C. The standard deviations of the data were within ± 5%, and the error bars are omitted for clarity (n = 4).

As HFIP and TFE were not chemically bound to TEOS, the greatly increased structure-inducing activities of the fluoro-alcohols in gel may be attributed to the confined environment in the porous gel, which may increase the local concentration of fluoro-alcohols around the protein in the gels, similar to the concentrating of urea around protein suggested by the data in Figure 5.1. Moreover, the increased structure influences of polymerized 3F or nPM suggest that the polymerization process as well as the spatial arrangements of the functional monomers may play important roles in enhancing the monomer-protein interactions in TEOS gel. Clearly, the strong MG-stabilizing activity of
3F is not lost upon co-polymerization with TEOS, while that of nPM is dramatically enhanced.

Although HFIP-induced maximum 1,8-ANS fluorescence in the gel was about 1.5 times higher than 3F, it is noteworthy that 3F was effective below half the concentration of HFIP, as inferred by fluorescence maxima at 0.17 M 3F versus 0.4 M HFIP. Moreover the MG transition window from 0.1 M to 0.2 M for 3F was much narrower than that for HFIP, which was between 0.15 M and 0.8 M. Therefore, the trifluoro-silane is a stronger protein structure inducer than the benchmark alcohol HFIP at low concentration range (< 0.3 M).

**Enzyme activity in 3F-modified gel**

Our fluorescence data have demonstrated that 3F-induced the changes of hydrophobic binding sites for 1,8-ANS in BCA. To further verify the influence of the trifluoro-silane on the protein conformation, the enzyme activity of BCA in TEOS gels modified with 3F or nPM was monitored at pH 7.5. As shown in Figure 5.6, relative to the enzymatic activity of BCA entrapped in pure TEOS, either 3F-TEOS or nPM-TEOS gels remained above 80% between 0.04 M and 0.11 M 3F / nPM. Beyond 0.11 M, 3F led to rapid loss of the enzyme activity, which was dropped to 30% at 0.2 M and below 10% at 0.24 M. In contrast, the enzyme activity remained above 65% from nPM-TEOS gels containing between 0.14 M and 0.24 M nPM. The enzyme activity data here clearly verify that polycondensed 3F and nPM in TEOS matrix both induced BCA structure transitions. The remarkable loss of enzyme activity induced by 3F (> 0.11 M) validates again that the trifluoro-silane has stronger influence on inducing protein conformation transitions than nPM, which parallel with the trend that the fluoro-alcohols HFIP and
TFE have superior effectiveness over other alkanols in evoking the structure transitions in proteins.\textsuperscript{19}

**Figure 5.6** The enzyme activities of 0.025 mg/ml BCA in TEOS gel balanced with 0.01 M pH 7.5 hepes buffer as a function of 3F or nPM concentration in TEOS gel at pH 7.5. TEOS concentration = 0.85 M – [3F] or [nPM]. The error bars represent the standard deviations of the data (n = 4).

BCA is overall spherical with a central beta-sheet consisting of 13 beta-strands and surrounded by 7 alpha-helices around the beta-sheet (Figure 5.7).\textsuperscript{37,38} The protein has two hydrophobic domains that are composed of the aromatic rings of Phe, Tyr and Trp residues from different beta-strands.\textsuperscript{38} The two hydrophobic clusters also play crucial roles in the stability and the catalytic activity of the enzyme.\textsuperscript{39} Thus, the 3F-induced 1,8-ANS fluorescence change and the enzyme activity loss are associated directly with the structure transitions of the two hydrophobic clusters, which could be the results from the destabilization / disruption of the beta-sheets in these domains.
Both 3F and TFE contain a tri-fluoro moiety that is linked by a short alkyl chain to an alcohol (TFE) or silanols (3F). The observation that 3F is a much stronger structure-inducer than TFE indicates a strong contribution from the alkyl-silanols, which was further supported by the MG-inducing activity of nPM, the non-fluorinated 3F analog. The preservation of activity following 3F and nPM condensation with TEOS suggests that not all three silanols participate in condensation, which is likely since condensation proceeds by formation of linear chains due to steric constraints around the alkyl- and tri-fluoro-substituted silanes. Moreover, the greater activity of 3F over nPM also points out the importance of the tri-fluoro groups, which enhance the structure-inducing activity cooperatively with the alkyl-silanol groups. Taken all together, our results support the application of 3F as a functional monomer for protein conformational imprinting, as well as numerous other applications where control of protein conformation at interfaces is desired.
CONCLUSIONS

The 3F-BCA interactions in the pre-polymerization complex and in TEOS gels were investigated to demonstrate the structure-inducing activity of the trifluoro-silane 3F. The protein structural freedom in TEOS gel was verified by the denaturation and renaturation of BCA in the gels, as inferred from challenges with urea either premixed or diffused in TEOS gel followed by elution. Hydrolyzed 3F-induced 1,8-ANS fluorescence changes in a time- and pH-dependent manner. In pH 2.5 hepes buffer two fluorescence peaks corresponding to MG-transitions evolved over 24 hours, while in pH 4 hepes buffer a single MG-transition peak was observed. These results at pH 2.5 and pH 4 demonstrate that BCA underwent sequential structure changes with the eventual loss of all protein secondary structure (F/F₀ close to 0) at high 3F concentrations. The solution data allow the concentration of hydrolyzed 3F or nPM to be tuned to maximally stabilize secondary structure prior to initiating condensation to entrap / imprint target proteins. The 3F-BCA interaction in the pre-polymerization complex was retained upon polymerization in TEOS gel, where single sharp peaks corresponding to MG-transitions were observed at 0.17 M 3F and nPM. The trifluoro-silane is demonstrated as a stronger structure inducer than nPM and HFIP, as inferred from that 3F-induced maximum 1,8-ANS emission was approximately 1.6 times of nPM, and it is effective at concentration less than half of HFIP and within narrower window (0.1 M - 0.2 M). Moreover, the enzyme activity assay at pH 7.5 verifies the strong transitions in 3F-TEOS gels, where BCA retained above 80% activity in the TEOS gel doped up to 0.11 M 3F, then dropped to approximately 30% activity for 0.2 M 3F and below 10% for 0.24 M. The structure-inducing activity of HFIP, and especially TFE, was enhanced against BCA entrapped in TEOS gels, supporting the
role of silica gels for priming proteins for selected structural transitions. The combined structure and enzyme activity data presented here clearly demonstrate the strong structure inducing activity of the trifluoro-silane 3F validating its use as a functional monomer for protein conformational imprinting.

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CHAPTER 6
CONCLUSIONS AND FUTURE WORK

CONCLUSIONS

In this report, the fluoro-silanes 3F and 7F were proposed as the functional monomers for construction of catalytic protein conformational imprint, which is capable of inducing structure transitions in template proteins. The preparation of sol-gel was first optimized in an attempt to obtain a biocompatible yet inert silica gel matrix for protein imprinting. A 3:1 ratio of sol to pH 8 hepes condensation buffer (0.01 M) led to rapid formation of transparent TEOS gels. 3F is demonstrated as a superior functional monomer to 7F for synthesizing optically transparent TEOS gel with uniform distribution of an encapsulated protein such as FITC-BSA.

Hydrophobic 3F dramatically influenced the structure of BLG in the pre-polymerization complex, leading to the formation of a MG-like intermediate at a concentration of only one-third of HFIP (0.2 M versus 0.6 M). Additionally, 3F-induced FRET in BLG was also threefold higher than that induced by HFIP, indicating the remarkable structure-inducing activity of hydrolyzed 3F in pre-polymerization complex. Furthermore, hydrolyzed 3F also induced the formation of a molten globule intermediate in BCA at a concentration of two-thirds of HFIP (0.4 M vs. 0.6 M). However, 3F-induced BCA structure transitions changed with time and were pH dependent. A stable MG-like intermediate was induced by hydrolyzed 3F at pH 4.5, while multiple formation of MG-like intermediate was formed at pH 2.5. The enzyme activity loss of BCA that encapsulated in TEOS gel was induced by either pre-mixed or diffused urea, and the removal of the denaturant led to enzyme activity recovery, validating the structure
freedom of the protein in TEOS gel. This recovery is an important prerequisite for functional monomers to induce protein conformational transitions. The interaction of 3F-BCA in the pre-polymerization complex was retained upon polymerization in TEOS gel, where 3F led to the evolvement of a MG-like structural transition of BCA at 0.15 M. The result is consistent with the enzyme activity transitions in 3F-TEOS gel, where 3F maintained ~ 80% of the enzyme activity near 0.17 M when the MG-like intermediate was formed from fluorescence data, beyond the concentration, the prompt decline of the probe emission accompanied up to 90% loss of enzyme activity at 24 hours.

Based on these results, the structure-inducing activities of 3F were demonstrated. The incorporation of 3F in TEOS gel retained the activity of hydrolyzed 3F in the pre-polymerization complex, validating the application of the trifluoro-silane as a functional monomer for the catalytic protein conformational imprinting.

**3F-PROTEIN INTERACTION MODEL**

Hirota et al. observed that both alkanediols (such as Et(OH)\textsubscript{2} and 2,3Bu(OH)\textsubscript{2}) and alkanetriol (such as glycerol) have profoundly weaker potentials than alkanols to induce alpha-helices.\textsuperscript{1} The increased number of hydroxyl groups was considered to reduce H-donor activity during the alcohol-protein interactions. Hence, they contribute negatively to the structure effects of TFE and HFIP. In our study, we also observed that the hydrolyzed TEOS, which owns four hydroxyl groups (Si-(OH)\textsubscript{4}), has almost no effect on structure transitions of the template protein. That is, the hydroxyl groups in TEOS may be more responsible for the dissolving of hydrolyzed silane in water instead of inducing the structure changes. Hydrolyzed nPM, the propyl substitution of TEOS, had significant structure effects on template proteins, suggesting the positive contribution of
propyl groups to the structure-inducing effects of 3F. The multiple hydroxyl groups in hydrolyzed 3F may contribute little to the induced protein structure transitions. However, they may still work very weakly as proton donors to form H-bonds with protein. In contrast, F atoms, which behave as better proton acceptors than H-donors, may actively interact with protein proton donor groups via the formation of H-bonds.

Moreover, our results have shown that the trifluoro groups and the alkyl group may contribute cooperatively to 3F-induced formation of MG-like intermediate that may largely be stabilized by increased content of alpha-helices. The hydrophobic interactions determined by these two groups, together with H-bonds formed between F and protein, may be the pivotal forces that dominate the process of 3F-protein interacting. Based on this understanding, we proposed a molecular model for 3F-protein interactions (Scheme 6.1). Due to strong hydrophobicity of the alkyl group and multiple F atoms, 3F may disrupt the hydration shell on the surface of the native protein, weaken the apolar interactions that stabilize the compact structure of the protein, and consequently lead to the protein unfolding and partially losing its tertiary structure. It is also possible that the hydrophobic moiety of 3F silanols may self-assemble, producing even stronger hydrophobic interactions and leading to greater solvent-exposure of protein hydrophobic residues. In the mean time, proton donor groups on protein may form H-bonds with F groups of 3F. Driven by these interactions, the distant H-bonds on polypeptide chain may be disrupted, and the intra-polypeptide hydrogen bonding is favored, leading to the formation and / or stabilization of helical structures. Although the tertiary structure of the protein is partially lost (hence molten), the compact secondary structure is largely maintained (hence globule) and a molten globule intermediate may thus be induced.
Scheme 6.1  Model showing 3F-protein interactions and 3F-induced protein unfolding and formation of alpha-helix based on a two-state transition mechanism. (a) The hydrolyzed 3F is represented schematically as consisting of hydrophilic silanol groups (≡Si-OH) and hydrophobic groups (–CH and –F). The composition of 3F allows it to act both as hydrogen donor (≡Si-OH) and as acceptor (−F). O is colored red, H is colored grey, C is colored black, Si is colored yellow and F is colored green. 3F may form H-bonds with protein via the hydroxyl groups (weak) and F groups (strong). (b) The native state protein with hydration shell outside the molecule. R represents different hydrophobic side groups on the peptide chain; D represents the amino acid with a hydrogen donor group. The hydrophobic side chains extend toward the protein hydrophobic core and non-local H-bonds form between beta-sheets. (c) 3F penetrates the hydration shell via hydrophobic interactions and forms H-bonds with the protein, leading to increased solvent exposure of hydrophobic residues. (d) The interactions between 3F and protein favor the formation of local H-bonds on the main chain, driving the formation and stabilization of alpha-helices. The hydrophobic interactions are presented as grey clouds.
To our knowledge, very few studies have been done on the physicochemical properties of the trifluoro-silane. Even less is known about 3F-protein interactions. The above molecular model is proposed based on our data and is from a structural viewpoint. Further investigations are necessary to obtain evidence associated with the detail of the model, for example 3F binding with protein and the aggregation of 3F silanol in solution.

**FUTURE WORK**

**Surface versus bulk imprinting**

In this work, 3F-induced global conformation transitions of proteins were demonstrated when template proteins were encapsulated in bulk silica gel. Bulk imprinting is the most straightforward approach for molecular imprinting. However, a key issue for bulk imprinting of protein is the limitation that the imprinted polymer imparts on protein movement. Thus, the kinetics of the protein adsorption / desorption process is unfavorable, and the mass transport becomes slow. The addition of a porogen into the imprinting material facilitates the exposure of binding sites and causes better controlled protein transport by forming macro-pores, where it leads to decreased polymer density, compromised mechanical stability (because of the reduced degree of cross-linking) and the loss of recognition and binding efficiency after a few rounds of regeneration. Environmental changes and additional requirements for harsh washing to remove the template also affect the function of the final materials. Consequently, the imprinting of larger proteins in bulk gel is practically limited, and alternative surface imprinting approaches become more favorable for protein-selective MIPs.

A fundamental strategy of surface imprinting is to locate the imprinted sites at or close to the surface of MIP, thereby enabling easy access of target protein molecules and
allowing specific proteins to be recognized for imprinting. Surface imprinting may cause decreased selectivity because only part of the protein is bound and recognized, and the site heterogeneity for such systems may also be high. However, those limitations may be outweighed by protocol modification. The advantages, including high mass transfer, less restricted binding kinetics, easy integration with sensor platforms and robustness gained by having a support, have made surface MIP the most preferable approach for protein imprinting.\(^3\)\(^-\)\(^5\) Some of typical surface imprinting approaches are presented in Table 6.1.

A widely applied surface imprinting method is to coat a thin film of protein-imprinted polymers on a flat surface or as a shell layer on the core of a micro / nano-sphere. Materials such as mica, silica, glass and polystyrene have been used frequently as support surfaces. A relatively easy process is to mix the template directly with the monomer solution and let the mixture polymerize on a surface. Proteins such as lactoperoxidase, Hb, horseradish peroxidase, cyt c and microperoxidase and CRP have been imprinted by this method in thin films.\(^6\)\(^-\)\(^9\) Glycoprotein transferrin (Tf) and GO have also been MIPed as free templates during the polymerization of core-shell micro-spheres.\(^10\)\(^-\)\(^12\)

Moreover, immobilization of template was exploited by many groups to promote protein recognition. In this method, template protein was not free in the solution but was first covalently anchored to a surface that was modified with aldehyde groups to form imine bonds with amino groups on the protein. A layer of polymer made from cross-linkers was then formed on the template-locked surface, followed by removal of the template and the rebinding of target protein. (Examples shown in Figure 6.1 and 6.2) As compared with imprints formed by free templates, significantly increased homogeneity
<table>
<thead>
<tr>
<th>Matrix/Cross link monomer</th>
<th>Surface/Support</th>
<th>Functional monomer</th>
<th>Template</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>poly-aminophenylboronic acid</td>
<td>layer on polystyrene microliter plate</td>
<td>poly-aminophenylboronic acid</td>
<td>lactoperoxidase, hemoglobin, horseradish peroxidase, microperoxidase</td>
<td>8</td>
</tr>
<tr>
<td>hexafluoropropylene</td>
<td>mica</td>
<td>disaccharide</td>
<td>albumin, lysozyme immunoglobulin G, ribonuclease, streptavidin</td>
<td>13</td>
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<tr>
<td>polyethylene glycol 400 dimethacrylate</td>
<td>glass cover slip</td>
<td>O-4-nitrophenylphosphorylcholine</td>
<td>C-reactive protein</td>
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<td>acrylamide and acrylic acid or acrylamide and N, N-dimethylaminopropylacrylamide</td>
<td>silica beads with amino groups (25-40 μm in diameter)</td>
<td>same as the matrix monomer</td>
<td>lysozyme</td>
<td>14</td>
</tr>
<tr>
<td>acrylic acid derivatives, (N,N'-1,2 dihdroxepthylene-bis (acrylamide), N,N'-(methylene)-bisacrylamide</td>
<td>acryl amide surface activated silica beads (10 μm in diameter)</td>
<td>same as the matrix monomer</td>
<td>glucose oxidase</td>
<td>11</td>
</tr>
<tr>
<td>acrylamide mixed with acrylic acid</td>
<td>silica beads activated with vinyl groups (25-40 μm in diameter)</td>
<td>same as the matrix monomer</td>
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<td>12</td>
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Table 6.1 (Continued)

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<th>Matrix/Cross link monomer</th>
<th>Surface/Support</th>
<th>Functional monomer</th>
<th>Template</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-aminopropyltrimethoxysilane and propyltrimethoxysilane</td>
<td>silica beads with aldehyde groups (average 110 μm in diameter)</td>
<td>same as the matrix monomer</td>
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<td>polyacrylamide gel beads (50 μm-100 μm in diameter)</td>
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<td>Staphylococcus aureus protein A</td>
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<td>Fe$_3$O$_4$ magnetite nanoparticles with aldehyde groups (18 nm in diameter)</td>
<td>methyl methacrylate (MMA)</td>
<td>bovine serum albumin</td>
<td>18</td>
</tr>
<tr>
<td>3-aminopropyltrimethoxysiloxane and TEOS</td>
<td>polysaccharide cross-linked from high-density chitosan (20 μm in diameter)</td>
<td>same as the matrix monomer</td>
<td>bovine serum albumin</td>
<td>19</td>
</tr>
<tr>
<td>N,N-methylenebisacrylamide</td>
<td>nano-porous alumina membrane</td>
<td>acrylamide</td>
<td>albumin, hemoglobin, cytochrome c</td>
<td>20</td>
</tr>
<tr>
<td>Matrix/Cross link monomer</td>
<td>Surface/Support</td>
<td>Functional monomer</td>
<td>Template</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------</td>
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<td>polystyrene sulphonate doped poly (3, 4-ethylenedioxythiophene)</td>
<td>polycarbonate membrane (PCM)</td>
<td>same as the matrix monomer</td>
<td>fluorescently labeled avidin (AV-FITC), tetrapeptide Tyr–Pro–Leu–Gly–NH2 (YPLG) (for recognition of oxytocin)</td>
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<td>ethylene glycol dimethacrylate</td>
<td>20-45 μm fraction from ground and sieved bulk gel</td>
<td>methacrylic acid</td>
<td>octapeptide Sar(1),Ala(8),AlaB angiotensin II (SA)</td>
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</tr>
<tr>
<td>ethylene glycol diacrylate</td>
<td>20-45-μm fraction from ground and sieved bulk gel</td>
<td>sodium acrylate,</td>
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<tr>
<td>ethylene glycol dimethacrylate</td>
<td>poly-EGDMA beads</td>
<td>N-methacryloyl-(L)-histidine-Cu$^{2+}$</td>
<td>L-histidine (for recognition of cyt c, ribonuclease A and L-histidine)</td>
<td>24</td>
</tr>
<tr>
<td>acrylamide, N, N-ethylene-bis-acrylamide and polyethylene glycol 200-diacrylate</td>
<td>a glass or oxidized silicon surface</td>
<td>same as the matrix monomer</td>
<td>C-terminus nonapeptide of three proteins (cytochrome c, alcohol dehydrogenase and bovine serum albumin</td>
<td>25</td>
</tr>
<tr>
<td>tetraethoxysilane</td>
<td>cylindrical silica scaffolds (4mm× 9mm): grounded bulk</td>
<td>γ-aminopropyltriethoxy siline (APS)</td>
<td>lysozyme and lysozyme C peptide (16 residues)</td>
<td>26</td>
</tr>
</tbody>
</table>
of binding sites has been achieved via template immobilization. The imprints of immunoglobulin G (IgG), Lyz and RNase, Hb, HSA and BSA by this way have demonstrated increased template recognition ranging from four- to 300-fold over their competitive controls. Several advantages such as rapid MIP synthesis, low amounts of monomers, solvent compatibility with template and the possibility to form template recognition sites in a solvent-free system, were also demonstrated.

Figure 6.1 Schematic representation for CRP micro-contact imprinting and rebinding. (a) Protein was adsorbed onto the microscope cover glass together with the functional monomer; (b) The cover glass was brought into contact with the support carrying cross-linker and initiator and then placed in a UV reactor; (c) The cover glass was removed; (d) Template protein was extracted by washing; (e) Rebinding of template or competitor proteins prior to ELISA reading (Adopted from Ref. 7).

In most cases, the core of the micro-sphere in surface imprinting is usually used as the support for the polymeric shell layer. The modification of core material has been explored by some groups in order to widen the scope of application. For example, Tan
Figure 6.2 Schematic representation for synthesis of protein-imprinted siloxane polymer on chitosan microsphere covalently immobilized with protein template (Adopted from Ref. 19).

magnetite (Figure 6.3), which brought about novel properties for the imprinting material such as easy isolation from a mixture or simplified transportation by employing an external magnetic field.\textsuperscript{18} Additionally, it makes it possible to apply the imprint as an affinity adsorbent in protein purification or as a probe for tumor imaging through magnetic resonance imaging.

Because the imprinted sites are suited at or close to the surface for surface imprinted material, template removal is largely simplified, and accessibility of target protein is greatly enhanced. However, protein binding capacity in surface imprinted materials is always lower compared to bulk imprinting. To solve this problem, imprinting on support structures down to nano-scale has been explored to obtain greatly increased surface area.\textsuperscript{20,21} To do this, a nano-scale sacrificial support such as nano-porous alumina or polycarbonate membrane (PCM) with track-etched pores is usually used to absorb the template. Then, matrix is polymerized in the pores. After removing sacrificial support and
template, surface imprinted polymer nano-wire or nano-rod were thus synthesized. (Example is shown in Figure 6.4.)

In recent years, an alternative surface imprinting method named “epitope” imprinting has been developed by Rachkov and co-workers.\textsuperscript{22,23,31} It was inspired by epitope recognition and binding from cell-cell interaction. In this method, a short peptide sequence exposed at the protein surface (as the epitope of an antigen), instead of the

![Figure 6.3](image_url)  
**Figure 6.3** Surface imprinting of protein on magnetic particles by covalent immobilization of template protein. The super-paramagnetic polymeric core was aldehyde modified and the protein template (BSA) was immobilized by amino acids, forming imine bonds with the aldehyde groups. The cross-linkers were then polymerized as the shell layer, after template removal by alkaline hydrolysis, binding cavities selective to BSA formed on surface of the imprints (Modified from Ref. 18).
whole protein, is imprinted as the template. Once the matrix has been polymerised, the resultant imprinted material should be able to recognize and bind the entire protein. (Example is shown in Figure 6.5.) Owing to the low number of potential binding sites for non-target protein, the non-specific binding that could easily be a problem for other surface imprinting methods is largely reduced in epitope imprinting.

The epitope imprinting method revealed broad success for imprinting of a variety of molecules in polymers.\textsuperscript{32,33} Chemical synthesis of peptide epitope is inexpensive, and a commercial product based upon a concept similar to Rachkov’s epitope approach has been recently launched.\textsuperscript{34} Concerning protein recognition, successful imprints have been reported by imprinting short peptides for reorganization of globule proteins.\textsuperscript{22-24,26,35}

![Diagram of nano-rods protein imprinting on a gold electrode](image)

**Figure 6.4** Schematic representation of the nano-rods protein imprinting on a gold electrode (Adopted from Ref. 21)
However, it is still difficult to evaluate the true efficiency of the epitope approach since the epitope exploited in experiments reported thus far were up to a few amino acids long. Also, the templates that they were intended to mimic were only several amino acids long, lacking significant three-dimensional structures. No simple and efficient method has been built for proteins greater than 100 amino acids. Therefore, the recognition of epitope imprints toward high molecular weight proteins still faces many challenges.

Figure 6.5 The protocol for epitope imprinting of proteins on glass or oxidized silicon surfaces. A) Glass surface modification and peptide attachment at N-terminal: the vinylsilane surface was oxidized with KMnO₄, NaIO₄, and K₂CO₃, followed by the activation of carboxylic acid-terminated monolayer with NHS/EDC (N-hydroxysuccinimde/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide). The peptide epitope were subsequently attached to the substrate in a solution of phosphate buffer and NaCl. B) MIP film fabrication and evaluation: The C-terminus nonapeptide epitope is attached through a tether to a glass or oxidized silicon surface by the N-terminal amino acid of the peptide. Monomers are photo-polymerized with a UV lamp, the glass substrate and/or silicon wafer was then removed by soaking the film and the substrate in buffer, whereupon the film and substrate spontaneously separated. C) The recognition of C-terminus peptide-sequence-imprinted surfaces toward proposed target protein by many cooperative weak interactions such as hydrogen bonds and hydrophobic interactions (Adopted from Ref. 28)
**Future directions**

Future efforts of this research will be focused on the exploration of the surface imprinting strategies that can provide proper target accessibility and easy polymer regeneration. More importantly, appropriate application platforms will need to be built for this protein conformational imprinting.

In order to be used as a plastic enzyme mimic to induce structure transitions or to correct misfolded protein conformation, a biocompatible and biodegradable matrix with good stability and longer shelf time needs to be developed. In this aspect, synthetic hydrogels usually have well-defined structures that can be modified to yield tailorable degradability and compatibility. Degradable hydrogels have already been successfully employed in numerous applications because of their unique properties. In particular, stimuli responsiveness that offers facile modulating for both protein binding and release can be easily tailored into hydrogel networks during fabrication. For example, temperature-sensitive hydrogels, which respond to external temperatures and undergo reversible swell-collapse transitions, can be fabricated from N-isopropylacrylamide (NIPAAAM) and polypropylene oxide-polyethylene oxide-polypropylene oxide (PPO-PEO-PPO) block copolymers.36-40

Additionally, supporting systems with maximized surface area such as nanoparticles can be used to increase the binding capacity. Moreover, because nanoparticles that are made of gold, carbon nanotubes, or magnetic compounds have been known to preferentially absorb electromagnetic (EM) radiation ranging from radio frequencies to x-rays, producing thermal, acoustic or chemical energy, EM-activated energy can be applied as an external stimulus to trigger the polymer transitions coated on
Scheme 6.2 Schematic presentation of thermal response hydrogel imprinting coated on electromagnetic nanoparticle: electromagnetic energy activation of the nanoparticle core yields increased thermal energy, causing hydrogel swelling and subsequently leads to protein adsorption. Polymer de-swelling occurs upon decreasing the temperature and the protein releases from the imprint.

EM-absorbing nanoparticles (Scheme 6.2). EM-absorbing nanoparticles can also be used for effective control of the protein desorption.

Furthermore, many aspects of physicochemical properties of the surface imprinting need to be examined to obtain optimal protein adsorption / desorption. The ratios of 3F functional monomer and the extent of matrix crosslinking may be manipulated to obtain high selectivity and sensitivity. Due to the hydrophobic interaction between the protein and 3F functional monomer, temperature controlled protein
desorption strategy, such as rinsing with cold saline, can be used for easy release of the imprinted protein. Moreover, since each protein possesses a distinct tertiary / quaternary structure with exposed active amino acid residues, it is not surprising that we observed different extents of protein-polymer interaction in this work. Therefore, it is necessary to extend the future research to additional template / target proteins, especially the misfolded proteins that associated with conformational diseases.

Last but not least, the protein conformational MIP fabricated in this research aims at the biomedical applications including laboratory analysis, clinic diagnosis and / or correction of misfolded proteins. Future work may face many challenges related to its therapeutic applications. Experiments on bio-safety, internal bio-availability, tunable delivery strategies, controlled release profiles and internal metabolism have to be designed and carefully carried out to test the premise. In addition, it would also be interesting to explore additional applications for this plastic enzyme mimic MIP, such as the dissolution of protein aggregates in bioreactor, which is frequently a problem due to protein over-expression.

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APPENDICES
APPENDIX A

In this part, the complementary data on BSA encapsulation and conformation in fluoro-modified TEOS gel are presented. Figure A.1 presents the data on the gelation rate of TEOS gel that was prepared by stir-mixing (600 rpm) hydrolyzed TEOS sol with 0.01 M hepes buffer (pH ranging from pH 4 to approximately pH 8) at various volume ratios (4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4). The results showed rapid condensation for pH 7 and 8 buffers, whereas below pH 6, the condensation was very slow, taking at least 5 hours for gelation. With the addition of 0.1 M pH 8 hepes buffer, gels formed within 20 to 100 minutes, depending on the volume of condensation buffer added.

To investigate the interaction of 1,8-ANS with hydrophobic 3F or 7F in TEOS gel, 90 μM 1,8ANS was diffused in 200 μl protein-free TEOS gel or fluoro-modified TEOS gel. Fluoro-modified TEOS gels were prepared by mixing hydrolyzed 3F or 7F with TEOS sol at 5% or 10% ratios (mol%) in condensation mixture. Figure A.2 presents the fluorescence change of diffused 1,8-ANS until it reached an equilibrium in approximately 2 hours. Both 3F and 7F led to increasing of 1,8-ANS fluorescence, the extend of which was well consistent with the content and the hydrophobicity of the silane itself: 10% 7F > 5% 3F > 10% 3F > 5% 3F. The data were used as the blanks and were subtracted from the fluorescence of protein-contained sample gels (fluorescence of protein-bond 1,8-ANS in gel > 20 times that of probe binding with 7F).

Figure A.3 shows the quenching of BSA Trp fluorescence and emissions of 1,8-ANS induced by HFIP / TFE at concentrations up to 3.5 M. Data presented in Chapter 3 only show the fluoro-alcohol induced fluorescence transition up to 1.2 M in
order to match the concentration range of the hydrolyzed silane. The data were normalized at the ratio of the sample fluorescence to that of alcohol-free samples (F/F₀).
Figure A.1 The gelation time as the function of the pH of 0.01 M hepes condensation buffer that mixed with TEOS sol at the indicated volume ratios (sol:buffer). The standard deviations are within ± 5%; the error bars are omitted for clarity (n = 4).

Figure A.2 The changes of 1,8-ANS fluorescence with the time of diffusing in 3F or 7F modified silica gels. Each gel (200 μl) was protein free and diffused with 90 μM 1,8-ANS. The standard deviations are within ± 2%, and the error bars are omitted for clarity (n = 4).
Figure A.3 The Trp fluorescence of BSA and the fluorescence of 1,8-ANS binding with BSA in presence of HFIP and TFE in 0.01 M pH 2.5 hepes buffer. BSA mixed with 1,8-ANS at 4.5 μM / 90 μM. The standard deviations are within ± 4%, and the error bars are omitted for clarity (n = 4).
APPENDIX B

Appendix B presents the fluorescence data on BLG-3F interaction in 3F-modified TEOS gel. 3F / nPM were doped in TEOS gel by mixing a hydrolyzed silane with TEOS sol in a condensation mixture at various molar ratios. The benchmark fluoro-alcohols HFIP and TFE were entrapped in TEOS by mixing with hydrolyzed TEOS sol in a condensation mixture at various volume ratios. The final pH of each gel was approximately pH 6. Each gel was formed within approximately 10 to 20 minutes of mixing and was aged in 4 ºC for 1- 2 hours before monitoring the fluorescence.

Figure B.1 shows BLG intrinsic fluorescence as a function of the concentration of the silanes (3F / nPM) or the fluoro-alcohols (HFIP / nPM) in TEOS gels. The results show that greatly enhanced Trp emission was induced by 0.17 M 3F or nPM. HFIP led to fluorescence quenching at 0.2 M, while TFE did not show any resolved influence on intrinsic fluorescence.

Figure B.2 presents the changes of HFIP / TFE-induced fluorescence intensity of BLG Trp residues in TEOS gel. The intrinsic fluorescence was monitored at the indicated time intervals after the gel formation. The results show that HFIP and TFE quenched Trp emissions at about 2% (v/v) and 7%, respectively. Further interaction between BLG and the fluoro-alcohols led to increased Trp fluorescence in the presence of HFIP < 3% or TFE < 10%. Beyond the concentration, the fluoro-alcohols further quenched BLG intrinsic emissions with time.

Figures B.3 and B.4 show the changes of 1,8-ANS fluorescence intensity with time in TEOS gels doped with the fluoro-alcohols (HFIP / TFE) and silanes (3F / nPM), respectively. The fluorescence was monitored after the probe was diffused and bound
with BLG in the gels at the indicated time intervals. From the results, the probe fluorescence intensity in all the gels increased with time.

The fluorescence spectrums of 1,8-ANS in TEOS gels doped with various concentrations of 3F or nPM are shown in Figures B.5 and B.6. Each gel was encapsulated with 20 μM BLG and diffused with 24 μl 835 μM 1,8-ANS. The probe fluorescence was excited at 295 nm, and the emissions from 400 nm to 600 nm were presented. 3F and nPM all led to the maximum 1,8-ANS fluorescence at 0.17 M. The maximum emission was at 480 nm when 3F or nPM was less than 0.11 M but shifted to 475 nm when the silanes concentration was greater than 0.11 M.
Figure B.1 The fluorescence intensity of 1,8-ANS binding with BLG in TEOS gel either entrapped with the fluoro-alcohols (HFIP and TFE) or copolymerized with 3F or nPM. Each gel encapsulated with 20 μM BLG and diffused with 24 μl 835 μM 1,8-ANS. The data were normalized as the ratio of the sample fluorescence to the cosolvent-free control (F/F₀). The standard deviations are within ±5%, and the error bars are omitted for clarity (n = 4).
Figure B.2 The dependence of fluorescence intensity of BLG Trp residues on the concentration of fluoro-alcohols (HFIP / TFE) entrapped in TEOS gel. The fluorescence was monitored at indicated time intervals after the gels formed. Each gel encapsulated with 20 μM BLG. The standard deviations are within ± 5%, and the error bars are omitted for clarity (n = 4).
Figure B.3 The dependence of the fluorescence intensity of 1,8-ANS on the concentration of fluoro-alcohols (HFIP / TFE) entrapped in TEOS as the probe diffused in the gels at indicated time intervals. Each gel encapsulated with 20 μM BLG and diffused with 24 μl 835 μM 1,8-ANS. The standard deviations are within ± 5%, and the error bars are omitted for clarity (n = 4).
Figure B.4 The dependence of the fluorescence intensity of 1,8-ANS on doping concentration of 3F or nPM as the probe diffused in the gels at indicated time intervals. Each gel encapsulated with 20 μM BLG and diffused with 24 μl 835 μM 1,8-ANS. The standard deviations are within ±5%, and the error bars are omitted for clarity (n = 4).
Figure B.5 The fluorescence spectrum of 1,8-ANS in TEOS gel doped with various concentration of 3F. Each gel encapsulated with 20 μM BLG and diffused with 24 μl 835 μM 1,8-ANS. The probe fluorescence was excited at 295 nm and the emissions from 400 nm to 600 nm were presented. The maximum emission was at 480 nm when 3F < 0.11 M and shifted to 475 nm at concentration > 0.11 M.
Figure B.6 The fluorescence spectrum of 1,8-ANS in TEOS gel doped with various concentration of nPM. Each gel encapsulated with 20 μM BLG and diffused with 24 μl 835 μM 1,8-ANS. The probe fluorescence was excited at 295 nm and the emissions from 400 nm to 600 nm were presented. The maximum emission was at 480 nm when nPM < 0.11 M and shifted to 475 nm at concentrations > 0.11 M.
Appendix C presents the complementary data for the interaction between BCA and the silanes (3F / nPM) or the fluoro-alcohols (HFIP / TFE). Figure C.1 shows the dependence of BCA Trp fluorescence on the concentrations of hydrolyzed 3F / nPM in 0.01 M pH 4 hepes buffer. The changes of the emissions were monitored at the indicated time of the BCA-silane interaction. From the results, while nPM did not led to significant change on protein intrinsic emissions, rapid emission quenching was induced by the addition of hydrolyzed 3F and a greatly enhanced fluorescence was induced by 0.4 M 3F. The trifluoro-silane- or nPM-induced Trp fluorescence did not change significantly with the time when silane was less than 0.3 M; slightly fluorescence quenching was induced when silane was greater than 0.3 M.

Figure C.2 shows the dependence of BCA Trp fluorescence on the concentrations of cosolvents (hydrolyzed 3F / nPM, HFIP, TFE) in 0.01 M pH 2.5 hepes buffer. The fluorescence data was recorded after the mixed samples equilibrated at room temperature for approximately one hour HFIP led to a concentration-dependent fluorescence quenching. 3F only reduced Trp emissions at a concentration greater than 1M. In contrast, both TFE and nPM had no resolved influence on BCA intrinsic fluorescence.

In Figure C.3, the influences of various cosolvents (hydrolyzed 3F / nPM, HFIP, TFE) on 1,8-ANS fluorescence in pH 2.5 hepes buffer (0.01 M) were presented. The fluorescence data was recorded after the mixed samples equilibrated at room temperature for one hour. HFIP, hydrolyzed 3F or nPM all led to the formation of probe fluorescence peaks, while TFE slightly increased the probe emissions. The order of the cosolvent-induced maximum fluorescence intensity is as follows: HFIP > nPM > 3F. This
is consistent with the order of the effective concentration for each cosolvent: $0.6 \text{ M HFIP} > 0.4 \text{ M nPM} > 0.3 \text{ M 3F}$.

Figure C.4 presents BCA Trp fluorescence in TEOS gel either doped with HFIP / TFE or modified with 3F / nPM. The final pH of each gel was around pH 4. (As 1,8-ANS binding with BCA had very little fluorescence emission at pH > 6, the gel had to be prepared under acidic condition.) Both 3F and nPM led to a dramatic change of Trp emission at 0.17 M; HFIP exhibited a concentration-dependent quenching, while TFE had no resolved influence on protein intrinsic fluorescence in the gels.

Figures C.5 and C.6 present the changes of 1,8-ANS fluorescence as the probe is diffused in TEOS gel entrapped with HFIP or TFE. The gels were prepared following the same protocol as in Figure C.4. In Figure C.5, HFIP induced the maximum 1,8-ANS binding with BCA at 6% (v/v). TFE did not led to the formation of a fluorescence peak, but it increased the probe emissions at concentration greater than 6% (v/v). HFIP or TFE-induced probe fluorescence all increased with the diffusion of 1,8-ANS in the gels. Due to the FRET between Trp and 1,8-ANS, the intrinsic fluorescence (in Figure C.4) decreased upon BCA binding with diffused 1,8-ANS. From the results in Figure C.6, HFIP induced greater Trp-fluorescence quenching than TFE. Additionally, BCA intrinsic emissions increased with time when HFIP was less than 4% or TFE was greater than 5%, while higher fluoro-alcohol concentrations (HFIP > 4% or TFE > 5%) led to the reduction of Trp emissions.

Figure C.7 presents the dependent of 1,8-ANS fluorescence on pH of the 0.01 hepes buffer. Each sample contained 20 μM BCA. The results show that the probe emission was enhanced at low pH condition (pH 3) while it was almost completely
quenched in the buffer with pH greater than 5. Figure C.8 shows the fluorescence spectrum of 1,8-ANS in 0.01 M pH 3 hepes buffer. BCA was mixed with ANS at 20 μM / 100 μM in the sample. The maximum fluorescence was shown at 480 nm.

Figures C.9 and C.10 show the fluorescence spectrum of 1,8-ANS in TEOS gel that was doped with various concentrations of 3F and nPM, respectively. Each gel was encapsulated with 20 μM BCA and was diffused with 120 μl 334 μM 1,8-ANS. The probe fluorescence was excited at 295 nm, and the emissions from 400 nm to 600 nm were presented. Both 3F and nPM led to the maximum 1,8-ANS fluorescence at 0.17 M. Additionally, the maximum probe emission was at 480 nm for pure TEOS gel, while it shifted to 475 nm for all 3F-doped gels. In contrast, the maximum probe emissions were at 480 nm for all the gels with nPM modification except for the gel doped with 0.17 M nPM, in which the maximum fluorescence shifted to 475 nm.
Figure C.1 The effects of hydrolyzed 3F or hydrolyzed nPM on fluorescence of BCA Trp residues after interacting with the protein in 0.01 M pH 4 hepes buffer. The data was recorded at indicated time intervals of the sample mixing. Each sample contained 20 μM BCA. The data were normalized as the ratio of the sample fluorescence to the silane-free control (F/F₀). The standard deviations are within ± 4%, and the error bars are omitted for clarity (n = 4).
Figure C.2 The dependence of the fluorescence intensity of 20 μM BCA Trp residues on concentration of various cosolvents (HFIP, TFE, hydrolyzed 3F, hydrolyzed nPM) in 0.01 M pH 2.5 hepes buffer. The data were normalized as the ratio of the sample fluorescence to the cosolvent-free control (F/F₀). The standard deviations are within ± 5%, and the error bars are omitted for clarity (n = 4).
Figure C.3 The dependence of the fluorescence intensity of 1,8-ANS on concentration of various cosolvents (HFIP, TFE, hydrolyzed 3F, hydrolyzed nPM) in 0.01 M pH 2.5 hepes buffer. BCA was mixed with ANS at 20 μM / 100 μM in each sample. The data were normalized as the ratio of the sample fluorescence to the cosolvent-free control (F/F₀). The standard deviations are within ± 5%, and the error bars are omitted for clarity (n = 4).
Figure C.4 The fluorescence intensity of BCA Trp residues in TEOS gel either entrapped with the fluoro-alcohols (HFIP and TFE) or copolymerized with 3F or nPM. Each gel encapsulated with 20 μM BCA. The standard deviations are within ± 6%, and the error bars are omitted for clarity (n = 4).
Figure C.5 The dependence of the fluorescence intensity of 1,8-ANS on the concentration of fluoro-alcohols (HFIP / TFE) entrapped in TEOS gel as the probe diffused in the gels at indicated time intervals. Each gel encapsulated with 20 µM BCA and diffused with 120 µl 334 µM 1,8-ANS. The standard deviations are within ± 5%, and the error bars are omitted for clarity (n = 4).
Figure C.6 The dependence of fluorescence intensity of BCA Trp residues on the concentration of the fluoro-alcohols (HFIP / TFE) entrapped in TEOS gel. The fluorescence was monitored at indicated time intervals after the gels formed. Each gel encapsulated with 20 μM BCA and diffused with 120 μl 334 μM 1,8-ANS. The standard deviations are within ± 5%, and the error bars are omitted for clarity (n = 4).
Figure C.7 The pH dependence of 1,8-ANS fluorescence binding with BCA in 0.01 M hepes buffer. BCA was mixed with ANS at 20 μM / 100 μM in each sample. The error bars represent the standard deviation for the data (n = 4).

Figure C.8 The fluorescence spectrum of 1,8-ANS binding with BCA in 0.01 pH 3 hepes buffer. BCA was mixed with ANS at 20 μM / 100 μM in each sample.
Figure C.9 The fluorescence spectrum of 1,8-ANS in 3F-doped TEOS gel. Each gel encapsulated with 20 μM BCA and diffused with 120 μl 334 μM 1,8-ANS. The probe fluorescence was excited at 295 nm and the emissions from 400 nm to 600 nm were presented. The maximum emission shifted from 480 nm in pure TEOS gel to 475 nm in all 3F-doped gels.
Figure C.10 The fluorescence spectrum of 1,8-ANS in nPM-doped TEOS gel. Each gel encapsulated with 20 μM BCA and diffused with 120 μl 334 μM 1,8-ANS. The probe fluorescence was excited at 295 nm and the emissions from 400 nm to 600 nm were presented. The maximum emission was at 480 nm for all nPM-modified gels, but it shifted to 475 nm in the gel doped with 0.17 M nPM.
APPENDIX D

PERMISSION LETTER
Dear Dr. Turner,

I am in the process of preparing my dissertation in the Department of Biological Engineering at Utah State University. I hope to complete my degree in May of 2011.

I am requesting permission to include the attached paper, of which you are a coauthor, as a chapter in my dissertation. I will include acknowledgements to your contributions as indicated. Please advise me of any changes you require.

Please indicate your approval of this request by signing in the space provided, attaching any other form or instruction necessary to confirm permission. If you have any questions, please contact me.

Thank you,

Yun Peng

I hereby give permission to Yun Peng to use and reprint all of the material that I have contributed to Chapter 4 of his dissertation.

____________________________________
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CURRICULUM VITAE

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- Lecturer, Biochemistry, 1999 - 2005
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CONFERENCE PRESENTATIONS


RESEARCH PAPERS & MANUSCRIPTS


• **Y. Peng**, M. Walsh, D. Britt. *Trifluorosilane as functional monomers inducing structural transitions in bovine carbonic anhydrase in TEOS gel* (ready to submit to Journal of Molecular Recognition)


**AWARDS**

• 1st Place in Biomedical Poster Presentation at the Institute of Biological Engineering Regional Conference. Logan, Utah. December 4, 2009.

• 1st Place in Engineering Poster Session at 2009 Intermountain Graduate Research Symposium. Utah State University, Logan, Utah. April 6, 2009.

• 3rd Place in Biomedical Poster Presentation at the Institute of Biological Engineering Regional Conference. Logan, Utah. October 18, 2008.

• Platform Presentation Award at the Institute of Biological Engineering Regional Conference. Logan, Utah. October 18, 2008.

• 1st Place in Engineering Poster Session at 2006 Intermountain Graduate Research Symposium. Utah State University, Logan, Utah. April 5, 2006.