MAGDA DOLSKA. In Vitro Selection of RNA Sequences that Mediate the Formation of Iron Oxide Nanoparticles. (Under the direction of Professor Daniel L. Feldheim).

RNA sequences have been discovered to mediate the formation of magnetic cobalt-doped iron oxide nanoparticles. RNA \textit{in vitro} selection with magnetic field partitioning was used to evolve an initial library of \( \sim 10^{14} \) unique RNA sequences. Both native and imidazolyl modified RNA combinatorial libraries were subjected to the selection pressure. After eight rounds of the selection, imidazolyl modified and native, families of active RNA sequences were identified. In contrast to conventional metal oxide colloid preparations, which typically require elevated temperature, basic pH, and excess surfactant, iron oxide nanoparticles were formed at room temperature and neutral pH, and at low metal precursor concentration (FeCl\(_2\) 150 \(\mu\)M, CoCl\(_2\) 37.5 \(\mu\)M). Relative to metal precursor, the RNA concentration was significantly lower (0.9 \(\mu\)M), yet crystalline iron oxide nanoparticles were formed rapidly (1 minute) and with a narrow size distribution (2.9 ± 0.9 nm for imidazolyl modified isolate, 2.7 ± 0.5 nm for native isolate). The average iron to cobalt ratio was determined to be 28 : 1 for the modified RNA isolates and 21 : 1 for native RNA isolates.

The material synthesis was shown to be RNA and sequence dependant. The isolate I2_96 truncation experiments have showed that isolate I2_96 with 15, 24 or 54 bases removed from its 3’ end still retained its activity in mediating the formation of magnetic iron oxide nanoparticles. However the synthesized nanoparticles were smaller and the overall yield of the product was lower than for the full length sequence. Similarly in case of native isolate A5 the removal of 24 bases from its 3’ end decreased the yield and size of the synthesized nanoparticles. However, when most of the A5 sequence was removed leaving the conserved region with 6 bases on the 5’ and 3’ ends the ability to synthesize the iron oxide material was inhibited.

Various analytical techniques were employed in effort to identify the composition of the synthesized material. It was concluded that the synthesized material was a
magnetic iron oxide doped with cobalt; either magnetite (Fe₃O₄), maghemite (γ-Fe₂O₃) or a mixture of those.

In effort to generate more material the isolate A5, recovered from a native RNA in vitro selection, was genetically encoded into E. Coli via the pET16b plasmid. The resulting bacteria produced A5 RNA and when grown in presence of 1 mM of FeCl₂ and 0.1 mM of sodium citrate they generated spherical, iron containing nanoparticles, about 3 nm in size.
IN VITRO SELECTION OF RNA SEQUENCES THAT MEDIATE THE FORMATION OF IRON OXIDE NANOPARTICLES

by

MAGDA DOLSKA

A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

CHEMISTRY

Raleigh, North Carolina

2007

APPROVED BY:

Cair of the Advisory Committee

Dr. Daniel L. Feldheim

Dr. Brent T. Gunnoe

Dr. Edmond F. Bowden

Dr. Christian Melander

Dr. Scott A. Hale
DEDICATION

There are quite a few people I want to dedicate this thesis to. First to my wonderful parents, Alina and Zbigniew, they have raised me to be the person I am today. They have loved, guided and supported me, every step of the way. To my grandparents, Adela and Tomasz, they have been like second parents to me, giving me unconditional love and support. To my Godmother Elzbieta, who always believed in me and offered a helpful advice. To my siblings, Maciej and Marta, whom I love dearly. To my husband James, the love of my life, who has been there for me always, no matter what. Finally to our daughter Mila, the miracle of life, who inspires me every day.
BIOGRAPHY

Magda Dolska was born in Lapy, Poland on July 8, 1977 to Zbigniew and Alina Dolski. After graduating high school in Znin, Poland she went on to attend Adam Mickiewicz University in Poznan, Poland, where she earned a Masters of Science in Chemistry. In August of 2001, she continued her desire to broaden her education as a Ph. D. student in chemistry under the direction of Dr. Daniel L. Feldheim at North Carolina State University.
ACKNOWLEDGEMENTS

From the formative stages of this thesis, to the final draft, I owe an immense debt of gratitude to my “bad ass” supervisor, Dr. Daniel Feldheim. His sound advice and careful guidance were invaluable as I explored the potential of RNA in material chemistry. I would like to also thank Dr. Bruce E. Eaton, who as a collaborator always served with his expert advice and was never discouraged by temporary drawbacks. In addition, former and current members of both the Feldheim and Eaton research groups are appreciated for all of their assistance.

There are many colleagues whom I owe thanks to, for their help and support during my studies; Dr. Stella M. Marinakos for introducing me to nanoparticle synthesis and TEM; Dr. Lina A. Gugliotti for all training and help in the RNA lab; Dr. Stefan Kraemer for help with AFM/MFM; Dr. Dick Guenter for assistance with the HPLC; Dr. Tim Slit for helping me strategize the way to turn \textit{E. Coli} into iron oxide factories; Dr. Wallace Ambrose for always offering a helpful hand concerning TEM and STEM operation; Dom Zichi (SomaLogic) for providing me with DOSA sequence alignment program; Dr. Sofi Bin Salomon for patiently helping me with SQUID; Dr. Chris Ackerson for collaboration and continuing the studies on \textit{E. Coli} iron oxide factories.

A few people whom I met during my graduate studies have become more than colleagues to me. Not only have they been willing to help, they always served me with advice and scientific discussion. I am mostly grateful for their friendship and companionship. Those people are: Carly J. Carter, Virginia Burns, Chris Heinecke, Rachel Kudgus, Dr. Dana Didonato, Doug Young and Dr. Steven McCall.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>vii</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF SCHEMES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS AND TERMS</td>
<td>xii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
</tbody>
</table>

## References

### CHAPTER 1: SELECTION OF IMIDAZOLYL-MODIFIED RNA MOLECULES THAT MEDIATE THE GROWTH OF MAGNETIC IRON OXIDE NANOPARTICLES

1.1 Results and discussion

1.1.1 Selection process

1.1.2 Materials characterization

1.1.3 RNA studies

1.2 Summary

1.3 Experimental Section

1.4 References

### CHAPTER 2: SELECTION OF NATIVE RNA MOLECULES MEDIATING THE GROWTH OF MAGNETIC IRON OXIDE NANOPARTICLES

2.1 Results and discussion

2.1.1 Selection process

2.1.2 Materials characterization

2.1.3 RNA studies

2.2 Summary

2.3 Experimental Section

2.4 References
CHAPTER 3: ENGINEERING OF E. Coli TO PRODUCE MAGNETIC IRON OXIDE NANOPARTICLES

3.1 Results and discussion

3.2 Summary

3.3 Experimental Section

3.4 References

CONCLUSION

APPENDIX
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table 1.1</th>
<th>Fraction of RNA recovered during magnet partitioning versus no magnet partitioning</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.2</td>
<td>Incubation conditions for selection rounds 10 through 18</td>
<td>17</td>
</tr>
<tr>
<td>Table 1.3</td>
<td>List of interplanar spacings (d) calculated from SAED of isolate I2_96 and corresponding HKL plane symbols</td>
<td>22</td>
</tr>
<tr>
<td>Table 1.4</td>
<td>Iron to cobalt ratios for different isolates after different washing procedures</td>
<td>25</td>
</tr>
<tr>
<td>Table 1.5</td>
<td>Raw ICP-MS data</td>
<td>26</td>
</tr>
<tr>
<td>Table 1.6</td>
<td>Fraction of RNA recovered during partitioning with and without the magnet for isolate I2_96 truncates</td>
<td>30</td>
</tr>
<tr>
<td>Table 2.1</td>
<td>Fraction of RNA recovered during magnet partitioning versus no magnet partitioning</td>
<td>44</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Iron to cobalt ratios for different native isolates after different washing procedures</td>
<td>50</td>
</tr>
<tr>
<td>Table A1</td>
<td>Experimental and theoretical d values comparison for selected area diffraction from experiment 1, (hkl)(theoretical d value) (% error)</td>
<td>83</td>
</tr>
<tr>
<td>Table A2</td>
<td>Experimental and theoretical d values comparison for selected area diffraction from experiment 2, (hkl)(theoretical d value) (% error)</td>
<td>84</td>
</tr>
<tr>
<td>Table A3</td>
<td>Theoretical d values for different iron oxide species and sodium and potassium chlorides</td>
<td>84</td>
</tr>
<tr>
<td>Table A4</td>
<td>Theoretical d values for different iron oxide species and sodium and potassium chlorides</td>
<td>85</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td>------</td>
</tr>
<tr>
<td>Figure I1</td>
<td>Examples of structures assembled by biological systems via biomineralization. From the left: calcite formed by <em>Emiliana huxleyi</em>, the Orange-banded brittle star, the <em>Magnetospirillum magnetotacticum</em> showing chain of magnetite</td>
<td>1</td>
</tr>
<tr>
<td>Figure I2</td>
<td>Examples of RNA folding: (a) RNA folding Scheme, (b) tRNA with its electrostatic potential (red) and crystallographically observed Mg ions (green), (c) 3D structure of ribozyme</td>
<td>4</td>
</tr>
<tr>
<td>Figure I3</td>
<td>Examples of UTP modifications</td>
<td>5</td>
</tr>
<tr>
<td>Figure 1.1</td>
<td>Example of radioactively labeled RNA monitoring in Partitioning procedure (cycle 8). Diamonds represent RNA content in consecutive washes and the square represents RNA present in resuspension</td>
<td>13</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Selection monitoring. Fraction of RNA retained in partitioning step as a function of a selection cycle number</td>
<td>14</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>TEM images and size distributions of material synthesized by inidazol-modified evolved pool</td>
<td>15</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Gel shift analysis of the material synthesized in round 6 (A), 7 (B) and 8 (C). Lane 1 contains pure transcript, Lane 2 contains incubation solution partitioned without the magnet, Lane 3 contains incubation solution that was not partitioned, Lane 4 contains material magnet partitioned</td>
<td>16</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Dideoxy sequencing gel of round 8 (A) and round 14 (B)</td>
<td>17</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>Imidazolyl-modified RNA sequences capable of catalyzing the formation of magnetic cobalt iron oxide nanoparticles. The sequences are shown grouped into families related by semi-conserved regions (conserved regions shown in color)</td>
<td>18</td>
</tr>
<tr>
<td>Figure 1.7</td>
<td>Figure 1.7 Time dependant size distribution for isolate I 2_96</td>
<td>20</td>
</tr>
<tr>
<td>Figure 1.8</td>
<td>SAED of material synthesized by isolate I 2_96 separated on the magnet</td>
<td>21</td>
</tr>
<tr>
<td>Figure 1.9</td>
<td>XPS spectrum for magnet separated material synthesized by isolate I 2_96, A – the entire spectrum, B, C, D - high resolution spectrum of Co, Fe and O regions respectively</td>
<td>23</td>
</tr>
</tbody>
</table>
Figure 1.10  Gel shift analysis of the material synthesized by I2_96 truncates: Lane 1 – Truncate T1 transcript; Lane 2 – T1 incubated with Fe^{2+}, Co^{2+}; Lane 3 – T1 incubated without metals; Lane 4 – Truncate T2 transcript; Lane 5 – T2 incubated with Fe^{2+}, Co^{2+}; Lane 6 – T2 no metals; Lane 7 – Truncate T3 transcript; Lane 8 – T3 incubated with Fe^{2+}, Co^{2+}, Lane 9 – T3 no metals.

Figure 2.1  Selection monitoring. Fraction of RNA retained in partitioning step as a function of a selection cycle number.

Figure 2.2  Gel shift analysis of the material synthesized in round 7. Lane 1 contains pure transcript, Lane 2 contains incubation solution partitioned without the magnet, Lane 3 contains incubation solution that was not partitioned, Lane 4 contains material magnet partitioned.

Figure 2.3  TEM image and size distribution of material synthesized by native evolved pool.

Figure 2.4  Native RNA sequences capable of catalyzing the formation of magnetic cobalt iron oxide nanoparticles. The sequences are shown grouped into families related by semi-conserved regions (conserved regions shown in color).

Figure 2.5  Time dependant size distribution for isolate A 5.

Figure 2.6  EELS spectrum of single particle shown in insert A synthesized with isolate A 5.

Figure 2.7  Material synthesized by truncate A5T1.

Figure 2.8  Gel shift analysis of the material synthesized by A5 truncate T1: Lane 1 – Truncate T1 transcript; Lane 2 – T1 incubated with Fe^{2+}, Co^{2+}; Lane 3 – T1 incubated without metals.

Figure 3.1  The pET16b cloning region.

Figure 3.2  In vitro transcription products-B, A–A5 transcript control.

Figure 3.3  Denaturing PAGE of RNA harvested from following bacterial strains: 1– BL21(DE3), 2 – BL21(DE3)pLysS, 3 - BL21(DE3)pLysE. Lane 4 - A5 transcript generated in vitro.

Figure 3.4  A - TEM image of the material synthesized by BL21(DE3) transformed with the engineered plasmid pET16bH8RA5;
B – cryo-electron tomography of material synthesized by
BL21(DE3)pLysS transformed with pET16bHδRA5

**Figure 3.5**  EDS spectrum of nanoparticles isolated from BL21(DE3) bacteria...

**Figure 3.6**  Plasmid test digest; A – *Bgl* II digest, B – *Hxo* I digest, C – non
digested plasmid.
<table>
<thead>
<tr>
<th>Scheme</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheme I</td>
<td>Steps of general RNA <em>in vitro</em> selection</td>
<td>6</td>
</tr>
<tr>
<td>Scheme 1.1</td>
<td>Steps of RNA <em>in vitro</em> selection</td>
<td>12</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS, SYMBOLS AND TERMS

<table>
<thead>
<tr>
<th>Abbreviation, Symbol or Term</th>
<th>Explanation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>Amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>AMV</td>
<td>avian myeloblastosis virus</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CTP</td>
<td>cytidine 5'-triphosphate</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxyctydine 5'-triphosphate</td>
</tr>
<tr>
<td>ddATP</td>
<td>dideoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ddCTP</td>
<td>dideoxyctydine 5'-triphosphate</td>
</tr>
<tr>
<td>ddGTP</td>
<td>dideoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>ddNTP</td>
<td>dideoxyribonucleotide 5'-triphosphate</td>
</tr>
<tr>
<td>ddTTP</td>
<td>dideoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>DOSA</td>
<td>daughter of sequence alignment</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine 5'-triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide 5'-triphosphate</td>
</tr>
<tr>
<td>ds</td>
<td>double stranded</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine 5'-triphosphate</td>
</tr>
<tr>
<td>EDS</td>
<td>energy dispersive x-ray spectrometry</td>
</tr>
<tr>
<td>EELS</td>
<td>electron energy loss spectroscopy</td>
</tr>
<tr>
<td>FEG</td>
<td>field emission gun</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineanesulfonic acid</td>
</tr>
<tr>
<td>HR-TEM</td>
<td>high-resolution transmission electron</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma mass spectroscopy</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani MCE mercaptoethanol MW molecular weight [Ni(PPh₃)₄] tetrakis(triphenylphosphine) nickel(0)</td>
</tr>
<tr>
<td>LS</td>
<td>liquid scintillation</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleotide 5'-triposphate</td>
</tr>
<tr>
<td>NZY⁺</td>
<td>NZ amine (casein hydrolysate) yeast extract</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>rATP</td>
<td>ribosomal adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SAED</td>
<td>selected area electron diffraction</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SQUID</td>
<td>superconductive quantum interference device</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine 5'-triphosphate</td>
</tr>
<tr>
<td>*UTP</td>
<td>5-(4-pyridylmethyl)-uridine 5'-triphosphate</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>XPS</td>
<td>x-ray photoelectron spectroscopy</td>
</tr>
</tbody>
</table>
INTRODUCTION

In nature biological organisms have been found to generate a wide variety of inorganic materials.\(^1\) During biomineralization protein enzymes act as catalysts of inorganic reactions, mediate the formation of growing crystals and control materials self-assembly to produce bone, teeth and shells.\(^2,3\) In addition to affording an organism structural integrity and protection against predation, many oxide materials found in nature have more sophisticated physical properties such as magnetism and light focusing.\(^4,6\) Some examples of biomineralization are shown in Figure I1: the *Emiliana huxlei* that mineralizes CaCO\(_3\) utilizing polysaccharides and proteins to nucleate and accumulate the mineral; the brittle star *Ophiocoma wenditii* which assembles microlenses from single anisotropic calcite crystals to form photonic structures; the magnetotactic bacteria which produces nanosized (25-120nm), single magnetic domain, well crystallized magnetite nanoparticles, with distinctive morphologies.\(^9\)

![Figure I1 Examples of structures assembled by biological systems via biomineralization. From the left: calcite formed by *Emiliana huxleyi*, Orange-banded brittle star, *Magnetospirillum magnetotacticum* showing chain of magnetosomes in the insert.](image)

Evolution and natural selection have resulted in biological systems capable of synthesizing metal oxides with controlled compositions, microcrystalline shapes, and long-range organization.\(^10\) A particularly interesting and ubiquitous metal oxide found in nature is magnetite, Fe\(_3\)O\(_4\). Magnetite is not only synthesized by magnetotactic bacteria, but also by single-celled algae and protists.\(^11-13\) Magnetite nanoparticles have been also found in higher organisms like salmon, trout and even in the human brain.\(^14-16\) Unlike geothermal formation or laboratory preparations of metal oxides, which typically require conditions of elevated
temperature, pressure, and pH, many organisms have evolved to catalyze metal oxide formation at temperatures close to 0 °C, under ambient pressure, and at neutral pH.\textsuperscript{17-19}

In an attempt to mimic biomineralization many scientists began studying proteins and polypeptides as templates and catalysts for material synthesis.\textsuperscript{20-23} Phage display techniques have been used to find peptides that interact with a variety of materials.\textsuperscript{24} This led to the exploration of engineered viruses to nucleate and template semiconductor nanoparticles into highly oriented quantum dot nanovires.\textsuperscript{25} Some research has been also focused on the interaction between materials and nucleic acids. Binding of dsDNA to CdSe quantum dots have been studied.\textsuperscript{26} The hybridization of DNA has been utilized to assemble gold nanoparticles into network structures\textsuperscript{27,28} and dsDNA has been used as metallization template.\textsuperscript{29}

This doctoral dissertation will focus on the synthesis of iron oxide nanoparticles using RNA, and RNA containing chemical modifications. It will be shown that RNA sequences that mediate the formation of magnetic iron oxide nanoparticles can be isolated from a large (ca. $10^{14}$ unique sequences) random sequence RNA library. The sequences were isolated using a new magnetic field selection protocol, so that only sequences that were bound to nanoparticles that responded to a magnetic field were recovered. As the chemistry of iron is complex, a brief discussion of the fate of iron cations in water is described below.

**Aqueous Chemistry of Iron**

The iron oxides have been known for millennia. There are sixteen known iron oxides. They can be divided into two categories: oxides for example magnetite (Fe$_3$O$_4$), maghemite (γ-Fe$_2$O$_3$), and hematite (α-Fe$_2$O$_3$); and hydroxides including goethite (α-FeOOH), akaganeite (β-FeOOH) or ferric iron hydroxide (Fe(OH)$_3$). Such minerals were used originally as pigments for paints during the Paleolithic Era. Much later, they were used in the magnetic compass when it was invented in China in the 3rd century. This was the first application of magnetic iron oxides, also known as lodestones, used by early navigators to locate the magnetic north. The most abundant deposits of these stones were found in the district of Magnesia in Asia Minor, hence the mineral’s name became magnetite.

Today applications of iron oxide colloids include cell separations, diagnostics, magnetic information storage, chemical sensing and contrast enhancers for magnetic
resonance imaging.\textsuperscript{30-35} It is a technological challenge to acquire control over the nanoparticles sizes, dispersity, crystal polymorph, and macroscopic morphology. The most interest among iron oxides has been focused on the magnetic species; magnetite and maghemite.

The chemistry of iron in aqueous solutions is very complex and dependent upon pH, temperature, iron precursors used and their concentrations. Ferrous ions (Fe\textsuperscript{2+}) in water quickly oxidizes giving ferric hexaaqua complex [Fe(O\textsubscript{2}H)\textsubscript{6}]\textsuperscript{3+}. The hydroxylation of those species can be initiated by addition of a base (pH $\geq$ 8), thermolysis or hydrothermolysis. As a result the aqua-hydroxy complex is obtained [(H\textsubscript{2}O)\textsubscript{5}Fe(OH)]\textsuperscript{2+}. Next the aqua-hydroxy complex undergoes condensation by olation, resulting in dimer species connected by single or double ol (-OH-) bridges (see reactions 1 and 2).

\[
\begin{align*}
[(\text{H}_2\text{O})_5\text{Fe(OH)}]^2^+ + [\text{Fe(O}_2\text{H)}_6]^3^+ & \rightarrow [(\text{H}_2\text{O})_5\text{Fe(OH)}\text{Fe(H}_2\text{O)}_5]^5^+ + \text{H}_2\text{O} \quad (1) \\
2[(\text{H}_2\text{O})_5\text{Fe(OH)}]^2^+ & \rightarrow [(\text{H}_2\text{O})_5\text{Fe(OH)}_2\text{Fe(H}_2\text{O)}_5]^4^+ + 2\text{H}_2\text{O} \quad (2)
\end{align*}
\]

Products of the condensation form a gelatinous precipitate called ferric gel composed of oxyhydroxides with various degrees of hydration (ferrihydrates). Upon aging of the solution and depending on the pH goethite (pH $\geq$ 10) or hematite (5 $\leq$ pH $\leq$ 10) is formed. If condensation undergoes in acidic conditions mixed olation and oxolation (formation of –O– bridges) occurs giving rise to nucleation of lepidocrocite $\gamma$-FeOOH. In the presence of ferrous ions ferric ions form spinel oxide over a wide range of compositions, at pH $> 9$. When the Fe(II)/Fe(III) ratio is 0.5 magnetite (Fe\textsubscript{3}O\textsubscript{4}) is formed. Smaller quantities of Fe(II) (0.5 $\geq$ Fe(II)/Fe(III) $\geq$ 0.1) lead to similar structures but containing vacancies. Fe(II) deficient particles tend to be smaller in size and transform into goethite ($\alpha$-FeOOH) after a few weeks. Magnetite due to electron mobility in the crystal lattice can transform to maghemite ($\gamma$-Fe\textsubscript{2}O\textsubscript{3}). The iron oxide or oxyhydroxide composition can be further influenced by the presence of certain anions. For example precipitation of ferric chloride in solution by the addition of a base or thermolysis leads to initial synthesis of akaganeite ($\beta$-FeOOH) that transforms to hematite upon aging. However in high chloride concentration the recrystallization does not occur.\textsuperscript{36}

Typically, magnetic iron oxide nanoparticles can be prepared by co-precipitating ferrous and ferric ions in aqueous solution.\textsuperscript{37} However, variations in the molar ratio of Fe\textsuperscript{3+}
and Fe$^{2+}$ can often lead to complicated changes in the crystalline structures of the obtained material.$^{38}$ The pH and the ionic strength play critical roles in iron oxide nanoparticles preparations, they also affect the chemical composition of the nanoparticle surface.$^{39}$ The sol-gel and hydrothermal methods were proven to produce magnetic materials with increased crystallinity, however the obtained nanoparticles often exhibit poor solubility and dispersibility.$^{40}$ Other synthetic methods include thermal decomposition of various iron precursors yielding nearly monodisperse maghemite or magnetite nanocrystals soluble in organic solvents.$^{41-43}$ Water soluble magnetite nanoparticles also have been prepared by thermal decomposition using polar coordination solvents.$^{44,45}$

**Ribonucleic Acid as a Potential Biomineralization Template**

We believe that ribonucleic acid (RNA) has a great potential to be used in material synthesis. RNA is a biopolymer involved in transcription and translation of genetic information in living organisms. It consists of four nucleotides (ATP, CTP, GTP, UTP) joined by phosphodiester bonds. RNA is highly organized, its complementary base pairing: A-U, G-C, and also G-U, A-C interactions, direct RNA folding and create reproducibly a number of structural motifs that are conformationally distinct and dependant on nucleotide sequence (Figure I2).

![RNA folding Scheme](image1.png)

**Figure I2** Examples of RNA folding: (a) RNA folding Scheme, (b) 3D structure of ribozyme, (c) tRNA with its electrostatic potential (red) and crystallographically observed Mg ions (green).

Shape diversity and functionality of RNA can be further increased by introducing nucleotide modifications, which allow for incorporation of virtually any desired functional group into the RNA chain.$^{46,47}$ Modified nucleotides have been found to be well tolerated by T7 RNA polymerase and are readily incorporated in RNA molecule in the transcription
Another major advantage of using RNA to mediate the formation of materials is the ability to employ an *in vitro* selection approach (Scheme Ii). Nucleotide sequences that catalyze certain reactions or specifically bind to molecular targets can be selected from synthetic combinatorial RNA libraries. The libraries provide huge complexities of nucleotide sequence and structure (typically in the order of $10^{14}$). During the *in vitro* selection process a pool of RNA sequences that are completely randomized at specific positions is subjected to selection pressure for catalysis or binding. The functional RNA's are selected during the partitioning step. The partitioning in the selection of RNA sequences can be designed to take advantage of binding constant or catalytic reactivity. Selected active sequences are reverse transcribed and amplified as dsDNA that is used for subsequent *in vitro* transcription. This newly transcribed RNA pool is enriched in the functional sequences and is then subjected to selection in the next cycle. Multiple rounds of enrichment result in the exponential increase of the active RNA species until they dominate the population of the sequences. This methodology assumes that the random pool of RNA sequences contains species performing a desired function. The *in vitro* selection approach has been successfully utilized to isolate a variety of RNA aptamers for a number of proteins and other biological receptors, as well as to small organic molecules. Aptamers are nucleic acids that specifically bind to a target molecule with high affinity and specificity. Furthermore, an impressive range of RNA-catalyzed reactions has been described, including amide bond formations, Diels-Alder reactions, alkylation and acylations.
The selection of RNA sequences that mediate the formation of solid-state materials can in principle be driven by application of similar selection pressures. RNA sequences that bind to certain crystals, or that catalyze the formation of materials, can be isolated. For example, the \textit{in vitro} selection approach has been applied to find RNA sequences that mediate the formation of palladium nanoparticles starting with zerovalent palladium complexes. It was shown that a size-based in vitro selection could be used to find RNA sequences capable of mediating the formation of large hexagonal and cubic palladium crystals.\textsuperscript{59,60} These simple first examples of RNA catalysis to form inorganic particles only required that labile organic ligands be displaced from the zero-valent metal precursor in favor of forming metal-metal bonds.

Herein a property based \textit{in vitro} selection will be described. A selection pressure was applied that was not simply based upon the size of the nanoparticle that formed, but on a \textit{property} of the material formed - a magnetic moment. Thus, for an RNA sequence to be selected and amplified, it was required to assemble a magnetic metal oxide under ambient conditions.

It will be shown that RNA sequences capable of mediating the formation of magnetic iron oxide nanoparticles can be selected using a magnetic field selection pressure. This is the first time that an RNA selection has been driven by a desired materials property. This work is exciting because it prompts a number of new fundamental questions: How does RNA mediate the formation of metal-oxygen bonds?; Are there now or were there ever RNA sequences present in the biosphere that controlled the growth of the material?
References


Morse, D. E. *Proceedings of the National Academy of Sciences of the United States of


CHAPTER 1: SELECTION OF IMIDAZOLYL-MODIFIED RNA MOLECULES
THAT MEDIATE THE GROWTH OF MAGNETIC IRON OXIDE
NANOPARTICLES

It has been shown that a size-based in vitro selection could be used to find RNA sequences capable of mediating the formation of large hexagonal and cubic palladium-containing particles.\textsuperscript{1,2} These simple first examples of the RNA use to control the growth of inorganic particles showed that specific RNA sequences can be isolated from a large random RNA sequence pool that interact with a growing crystal in such a way as to control crystal shape. In this chapter it will be shown that RNA sequences that mediate the formation of magnetic transition metal oxide nanoparticles can be isolated from iterative cycles of selection and amplification. In contrast to prior work, the goal of the RNA \textit{in vitro} selection described here was to determine if RNA sequences could be isolated based upon their ability to mediate the formation of a material with a desired property.

1.1 Results and Discussion

1.1.1 Selection process

The hypothesis that a random sequence RNA pool can evolve to form materials with a desired property was investigated using magnetic field partitioning. The magnetic field was intended to isolate those sequences capable of interacting with metal ions in such a way as to mediate the formation of a solid particle capable of migrating in a magnetic field. This magnetic \textit{in vitro} selection was accomplished following the selection cycle shown in Scheme 1.1. The selection began with a chemically synthesized (ABI 391) library of $10^{14}$ unique ssDNA sequences, 87 bp in length, containing a center region of 40 bp, random in sequence, flanked by regions of defined sequence, at the 5’ and 3’ ends, to allow for amplification and other enzymatic steps in the selection process. The random ssDNA library was amplified by two cycles of the polymerase chain reaction (PCR) (step not shown in scheme) generating a dsDNA library. In step 1 T7 RNA polymerase was used to transcribe the dsDNA library into a ssRNA library containing ca. $10^{14}$ sequences.
During step 1, modified 5-(4-imidazolylmethyl)-UTP (*UTP) was introduced into the random RNA pool to provide additional metal coordination sites and proton shuttling mechanisms beyond those provided by the heterocyclic nitrogens present in native RNA. In addition, transcription products were body labeled with $[^{\alpha-32P}]$ ATP, for detection and quantitation purposes. The RNA library obtained was purified on a 10K molecular weight cut-off filter by washing four times with 1x buffer (Na$^+$, K$^+$, PO$_4^{2-}$ ions present to assure RNA stability through the washing procedure) and resuspended in deionized RNase free water. In step 2, the RNA library (900 nM) was incubated with the metal salts FeCl$_2$ and CoCl$_2$ at 150 µM and 37.5 µM respectively. Additionally KCl, NaCl (100mM each) and HEPES buffer 50mM were present (Scheme 3). The incubation was performed in aqueous solution for 5 hours at ambient temperature. RNA molecules that mediated the formation of magnetic nanoparticles and remained bound to them were separated from remaining inactive RNA and unused reagents using magnetic field partitioning. To ensure that RNA sequences that bound nonspecifically to the sides of the tube were not carried forward a counter selection step was introduced. Prior to magnetic partitioning the reaction mixture was transferred to a fresh tube, thus eliminating any RNA bound to the tube. For selection step 3 to be successful, RNA
sequences were required to either mediate the formation of magnetic particles and to remain bound to those particles, or to simply bind to particles formed spontaneously or by other RNA sequences. The selected RNA was reverse transcribed using AMV reverse transcriptase in step 4, to give a cDNA copy of the “winning” RNA sequences. The cDNA was amplified without purification by means of PCR. The PCR amplification completed the selection cycle and provided a dsDNA template enriched in the winning sequences and ready for T7 RNA polymerase transcription at the beginning of the next cycle.

Typically, it is impossible to select functional RNA molecules in a single selection cycle since they are represented only by a fraction of a starting pool. Inactive RNA sequences tend to get carried along, and therefore the selection cycle has to be repeated 8-10 times. In this case, a population of RNA molecules directing the growth of magnetic iron oxide particles was separated after eight rounds of the \textit{in vitro} selection.

During each cycle, the amount of $^{32}$P labeled RNA lost in each of the four wash steps of the magnetic field partitioning procedure was monitored by LS (liquid scintillation) counting. The RNA discarded consistently followed the trend shown in Figure 1.1 (diamonds). Most of the RNA was lost during the first solution removal step. During the washing procedure, the RNA content in each consecutive wash gradually decreased almost to zero. The amount of resuspended RNA retained due to binding to magnetic particles was always significantly higher than the contents of the last wash (Figure 1.1, square).

To estimate the efficiency of the magnetic field partitioning and to ensure the RNA was not retained in the test tube due to nonspecific binding, control incubations were carried out during rounds 5 through 8. The control incubations were washed without the magnetic field. The tube containing the reaction mixture was washed 4 times with 200 µL of 1x buffer (K$^+$, Na$^+$, PO$_4^{3-}$) and resuspended in 100 µL of dH$_2$O. An aliquot of resuspended material
was counted on LS and compared with the results obtained from magnet-partitioned incubations, Table 1.1. As the selection progressed from round 5 to round 8, an increased percentage of the input RNA was retained using the magnetic field. A slight increase in recovery percentage of the RNA was also observed in no magnet controls. It was most likely due to the presence of the sequences that were nonspecifically binding to the sides of the tube. However, the difference between the magnet and no magnet partitioning increased as the selection progressed, which suggested that certain RNA sequences were interacting with a material that responded to the magnetic field.

Table 1.1 Fraction of RNA recovered during magnet partitioning versus no magnet partitioning.

<table>
<thead>
<tr>
<th></th>
<th>Magnetic Field Partitioned</th>
<th>No Magnetic Field</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[% RNA recovery]</td>
<td>[% RNA recovery]</td>
</tr>
<tr>
<td>Round 5</td>
<td>0.001 ± 0.003</td>
<td>0.002 ± 0.004</td>
</tr>
<tr>
<td>Round 6</td>
<td>0.038 ± 0.009</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Round 7</td>
<td>0.057 ± 0.006</td>
<td>0.019 ± 0.004</td>
</tr>
<tr>
<td>Round 8</td>
<td>1.79 ± 0.03</td>
<td>0.19 ± 0.02</td>
</tr>
</tbody>
</table>

To monitor the selection progress RNA retained in partitioning step of each cycle was recorded and then plotted as a function of the cycle number (Figure 1.2). In rounds 1 through 5 the fraction of resuspended RNA was very small and no significant variation was observed. In rounds 7 and 8 the RNA recovery increased. Typically such an increase in recovery would suggest that the selection reached completion. For that reason the round 8 evolved pool was characterized and compared to the material synthesized by the starting random RNA pool using transmission electron microscopy (TEM). Material recovered from incubating the metal ions with the initial RNA sequence pool revealed only a few particles of undefined shape. Following 8 cycles of selection and amplification, however,
spherical nanoparticles were observed by TEM (Figure 1.3). The mean particle size was 9.7 nm with 10.2 nm standard deviation. Such broad size distribution was most likely the result of different particle nucleation and growth rates due to the presence of different sequences in the evolved pool.

![Figure 1.3 TEM images and size distributions of material synthesized by inidazol-modified evolved pool.](image)

To eliminate the possibility of spontaneous magnetic nanoparticle formation and to confirm the necessity of RNA for the particle formation a series of control experiments were conducted. Reactions in which Co$^{2+}$ and Fe$^{2+}$ were incubated alone or in solutions containing nitrogen heterocycles such as histidine monomer or histidine rich polypeptide CGWPE(H)$_{10}$ under identical reaction and magnetic field partitioning conditions yielded no nanoparticles, based on TEM analysis. This suggested that selected RNA sequences were involved in the generation of the spherical nanoparticles shown in Figure 1.3.

As a complement to characterization by TEM, beginning at round 6 an aliquot of material synthesized each round was analyzed on 6% denaturing polyacrylamide gel electrophoresis (PAGE). Figure 1.4 shows gel images in which lanes 1 through 4 correspond to pure transcription product, incubation solution washed without the magnet, unpartitioned incubation solution and magnet partitioned incubation solution, respectively. In lane 3 there is a pronounced gel shift versus the transcription product lane. Slower mobility on the gel was caused by the RNA binding to high molecular weight material. Material from that lane was not partitioned; it was loaded on the gel directly following the incubation. Lane 4 corresponds to incubation solution that was magnet partitioned. The observed gel shift suggests the RNA binding to high molecular weight material that responds to the magnetic field. As a control material partitioned without the magnetic field was also analyzed on the
el, lane 2. In rounds 6 and 7 there was no gel shift detected when partitioning without the magnet.

In round 8 however a low intensity band at the top of the gel was detected. One caveat must be mentioned with respect to the gels shown in Figure 1.4. The observation of “hanging lanes” in gels is a common occurrence in molecular biology. A hanging lane is defined as a lane in which a small amount of material (RNA, DNA, or proteins) remains at the top of the gel. The origins of this artifact are not well understood, but in this case a hanging lane caused by RNA not bound to nanoparticles would be difficult to distinguish from RNA bound to nanoparticles. Note, however, that in this work hanging lanes were not observed for the pure RNA transcript run on the same gel as RNA incubated with metal ions.

To determine whether the selection was completed at round 8 the RNA pool recovered as PCR product from round eight of the selection was bulk sequenced to determine whether any sequence convergence could be observed. The method we applied was dideoxy nucleotide sequencing, commonly called Sanger sequencing. The DNA molecule was terminated whenever a dideoxy nucleotide (ddNTP) was incorporated into the growing strand; the resulting denaturing gel is shown in Figure 1.5 A. The round 8 evolved RNA pool showed no distinguishable pattern, in the 40N random region, suggesting that the RNA pool
was still relatively random and therefore the selection was not yet completed. Ten more selection cycles were performed, during which the selection pressure was increased by decreasing the reaction and magnet incubation times. The modified selection criteria are shown in Table 1.2.

**Table 1.2 Incubation conditions for selection rounds 10 through 18.**

<table>
<thead>
<tr>
<th>Round #</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13, 14</th>
<th>15, 16</th>
<th>17, 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction time</td>
<td>Over night</td>
<td>1h 45 min</td>
<td>1h 45 min</td>
<td>1h</td>
<td>1h</td>
<td>20 min</td>
</tr>
<tr>
<td>Magnet time</td>
<td>7h</td>
<td>5h</td>
<td>5h</td>
<td>2.5h</td>
<td>1.5h</td>
<td>1.5h</td>
</tr>
</tbody>
</table>

Selection progress was again monitored by recording the amount of recovered RNA at every cycle. Applying more strict incubation conditions decreased RNA recovery. At round 14 a significant increase in RNA recovery was observed. In order to determine whether the sequences converged at that point dideoxy sequencing of the DNA pool from round 14 was performed. Figure 1.5 B shows the resulting gel image. Despite the increased selection pressure, no sequence convergence was observed. The material synthesized by the round 15 and 18 evolved pools was investigated by TEM. Samples contained particles and aggregates of non defined shape. These results led to the hypothesis that the selection pressure in rounds 9-14 was too stringent, and that functional sequences had been lost. Since the round 8 RNA generated a high abundance of spherical nanoparticles (Figure 1.3) we decided to spatially resolve and identify individual sequences from that round of the selection using standard cloning and sequencing procedure. It is likely that the selection was indeed completed at round 8, however the location of evolved conserved regions might have varied within the RNA sequences, making it impossible to observe any

![Figure 1.5 Dideoxy sequencing gel of round 8 (A) and round 14 (B).](image)
sequence convergence using the bulk sequencing method.\textsuperscript{3}

To identify the RNA sequences from the round 8 of the selection the dsDNA, PCR product, from Step 5 of this cycle of the selection was used in standard plasmid cloning procedures. The sequences obtained were genomically aligned and grouped into families related by regions of high sequence similarity (Figure 1.6). Cloning and sequencing led to 35 sequences and two families were identified. Family 1 was comprised of 29 sequences which shared seven nucleotide region of high sequence similarity UUAACAU and 19 of them also contained motif UAUAUUA. Sequence I23 occurred twice. The other 6 sequences shared the UAUAUUA motif and were grouped into Family 2. The remaining I2_51 orphan sequence did not share any primary sequence homology with either family.

Based on the sequence analysis it was concluded that in order for RNA to be active in magnetic nanoparticle synthesis it was not necessary for the selected sequences to maintain 100\% consensus sequence identity. One or two point mutations were commonly observed. Within the motif UUAACAU 80\% identity was maintained among sequences from Family 1, fifth and seventh positions were the most conserved (90\%). Within the motif UAUAUUA shared by 25 sequences 79\% identity was maintained among those sequences, the sixth position was the most conserved (88\%). As suspected previously the location of the regions of high sequence similarity were position independent, they were shifted with respect

<table>
<thead>
<tr>
<th>Family 1</th>
<th>Orphans</th>
</tr>
</thead>
<tbody>
<tr>
<td>188 UUAACGUUUAAACACCUAAUAUAUGAUAUUAUUUCACU</td>
<td>186 I2_51 AUUAGCGGCUAUAUCUACCUCGUACUGU</td>
</tr>
<tr>
<td>12, 35 UAAAAAUAUUCGAAUUAUUAAAGAUAUAAACAUACGCG</td>
<td>184</td>
</tr>
<tr>
<td>12, 93 UACACCUAUUUAUUCACUAAUAAACAUACGCG</td>
<td>197</td>
</tr>
<tr>
<td>12, 101 UCAAAAUAUUCUCUCAAUAUGCAAGCGAAGAACG</td>
<td>191</td>
</tr>
<tr>
<td>12, 96 CCACGUACAUAAACAUAAUUACACAUUACCUUACU</td>
<td>192</td>
</tr>
<tr>
<td>12, 19 AAGGCUAAUCUAAUAUUAUUAUUAUAACGACU</td>
<td>193</td>
</tr>
<tr>
<td>12, 20 CCACUUCUCAUCUAAUAUUAUUAUUAACGACU</td>
<td>194</td>
</tr>
<tr>
<td>12, 55 AUUCAACUCAACUAACUAACUAACUAACCUUACU</td>
<td>195</td>
</tr>
<tr>
<td>186 CUUACGUACAUAAACAUAAUUUACUUCUUACUACU</td>
<td>196</td>
</tr>
<tr>
<td>12, 59 UGAAUUGCUAACCUAAUACAAUGAUUAUAAACGACU</td>
<td>197</td>
</tr>
<tr>
<td>12, 64 AUAUGGCUIAAAGACUUAACUACGACU</td>
<td>198</td>
</tr>
<tr>
<td>12, 72 ACAAAAUAUUCGAAUUAUUAAAGAUAUAAACAUACGCG</td>
<td>200</td>
</tr>
<tr>
<td>170 ACAAAUAAUUIUACCUAAUAUAAGAAAUUAACAUACGCG</td>
<td>201</td>
</tr>
<tr>
<td>12, 63 AAAAAAUAUUCGAAUUAUUAAAGAUAUAAACAUACGCG</td>
<td>202</td>
</tr>
<tr>
<td>12, 11 UUUCAACGUAAUAUUAUUAUUAUUAACGACU</td>
<td>203</td>
</tr>
<tr>
<td>12, 48 ACCACAUAAUUAUUAUUAUUAUUAACGACU</td>
<td>204</td>
</tr>
<tr>
<td>12, 50 AUAACCAACAUAAUUAUUAUUAUUAACGACU</td>
<td>205</td>
</tr>
<tr>
<td>12, 51 AUUAGCGGCUAUAUCUACCUCGUACUGU</td>
<td>206</td>
</tr>
</tbody>
</table>
to each other in the RNA strands; which made it impossible to observe any sequence convergence on bulk sequencing gels (Figure 1.5 A, B).³

1.1.2 Materials characterization

After completing the selection (section 1.1.1) and conducting a series of control experiments it was apparent that during the selection process RNA sequences were isolated that mediated the formation of a material that responded to magnetic field. The next step was to characterize the material being synthesized. The following section focuses on characterizing the shape and size distribution of the colloid synthesized by chosen individual sequences and how the average particle size and dispersity changed as a function of reaction time. Another important feature that was investigated is the material composition.

Isolates I47, I101, I2_4, I2_96 were subsequently chosen as representative sequences, and their ability to mediate the formation of the particles was investigated by TEM. Each isolate showed spherical colloid formation. Size distribution as a function of reaction time was analyzed for isolate I2_96. Iron and cobalt precursors were incubated with the chosen isolate in a magnetic field for 1, 10, 100 minutes; followed by magnet partitioning. The resulting nanoparticles were analyzed by TEM (Figure 1.7). The average particle sizes and their standard deviations listed in Figure 1.7 were obtained based on analysis of minimum of 100 particles. Colloid formation was detected after only 1 minute of reaction. The particles formed were 2.9 nm in size with 31.9% dispersity. After 10 minutes the mean size of the nanoparticles reached 4 nm and the colloid dispersity increased to 48%. Looking at the nanoparticle histogram for 10 minute incubation it appeared that predominantly two sizes were forming, about 2 and about 6 nm. After 100 minutes of reaction generated nanoparticles were 8 nm in size with 43% dispersity. Growth mechanism analysis suggested that regardless of the reaction length coagulation was responsible for the particle formation.⁴,⁵ When more closely analyzing the particle histograms it could be observed that at each time point the evidence of particle coagulation was present. At 1 minute incubation most of the particles were 2 and 3 nm in size, however about 10% of the particle population was between 4-6 nm, which most likely was a product of the fusion of the smaller particles. After 10 minutes the number of particles that were 2 and 3 nm in size dropped
slightly whereas more of the larger 4-9 nm particles were appeared as a result of smaller nanoparticles coagulation. The ratio between those two sizes became about 50:50. Finally after 100 minutes the 2 and 3 nm particles disappeared, the size distribution shifted to larger sizes. Moreover the particle size dispersity increased, which implied mixed growth by coalescence and possibly the Ostwald ripening.

Figure 1.7 Time dependant size distribution for isolate I 2_96.
Isolate I 2_96 was chosen for further characterization. In an attempt to identify the synthesized material we performed selected area electron diffraction (SEAD). It was noted that combining RNA with FeCl₂, CoCl₂, NaCl, KCl and HEPES buffer in aqueous solution could result in any of the following species; magnetite (Fe₃O₄), hematite (α-Fe₂O₃), maghemite (γ-Fe₂O₃), ferric oxide (FeO), goethite (α-FeOOH) or akaganeite (β-FeOOH). It was also possible that sodium and/or potassium chloride could precipitate during the TEM grid preparation. Diffraction patterns from 3 different experiments were collected. The list of interplanar spacings calculated from the radius of diffraction rings and (hkl) assigned to them by indexing to Fe₃O₄ are presented in Table 1.3. Example of indexed diffraction pattern is shown in Figure 1.8. The calculated interplanar spacings (d) were not identical between experiments; this might be indicative of the presence of more than one composition. Possibly a mixture of iron oxide species was synthesized, which is a common phenomenon in low iron precursor concentrations. It was also possible that the magnetite nanoparticles were synthesized and they either partially or entirely oxidized over time. The experimental d values were compared to the theoretical values of the possible compositions listed above (see Appendix, Table A1 through A4). As can be noted it was impossible to unambiguously identify the synthesized material based on the diffraction data since the theoretical interplanar spacings for different iron oxide/hydroxide species were very similar.
Table 1.3 List of interplanar spacings (d) calculated from SAED of isolate I2_96 and corresponding HKL plane symbols.

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interplanar spacings</td>
<td>Assigned (hkl)</td>
<td>Interplanar spacings</td>
</tr>
<tr>
<td>4.116</td>
<td>(200)</td>
<td>2.7060</td>
</tr>
<tr>
<td>3.071</td>
<td>(220)</td>
<td>2.0413</td>
</tr>
<tr>
<td>2.676</td>
<td>(310)</td>
<td>1.7623</td>
</tr>
<tr>
<td>1.876</td>
<td>(420)</td>
<td>1.265</td>
</tr>
<tr>
<td>1.611</td>
<td>(511)</td>
<td>1.0675</td>
</tr>
<tr>
<td>1.527</td>
<td>(521)</td>
<td></td>
</tr>
<tr>
<td>1.202</td>
<td>(444)</td>
<td></td>
</tr>
<tr>
<td>1.085</td>
<td>(553)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Another technique employed to identify the material synthesized by selected RNA sequences was X-ray Photoelectron Spectroscopy (XPS). Isolate I 2_96 was chosen as a representative of the most abundant family of the evolved pool. The sample was sputtered for 30 minutes to remove impurities. The resulting XPS spectrum, Figure 1.9, (referenced to adventitious carbon 285 eV) confirmed the presence of iron (bands at 710.2 eV and 723.7 eV) and cobalt (bands at 783.4 eV and 798.7 eV). The observation of a single oxygen 1s band at 532.2 eV in the XPS spectrum rather than two bands ruled out the formation of a hydroxide phase. Also no chloride band was detected which ruled out the possibility that the examined material was simply FeCl₂, CoCl₂, NaCl or KCl. It has been shown that sputtering can cause the conversion of iron species to FeO (710 eV), therefore the only conclusion that could be made from the XPS analysis, was that this material was comprised of an oxide containing both iron and cobalt.
Figure 1.9 XPS spectrum for magnet separated material synthesized by isolate I 2_96, A – the entire spectrum, B, C, D - high resolution spectrum of Co, Fe and O regions.
The XPS analysis indicated the presence of both iron and cobalt in the synthesized nanoparticles. It was then of interest to determine the molar ratio of those elements. In order to do that the synthesized material was analyzed by Inductively Coupled Plasma – Mass Spectrometry (ICP-MS). The resulting Fe : Co ratio, for different isolates, ranged from 24 : 1 to 32 : 1, Table 1.4. The raw ICP-MS data is presented in Table 1.5. To address the possibility that Co$^{2+}$ ions were simply ion paired to the polyanionic backbone of RNA or coordinated to the imidazolyl moieties, particles prepared with RNA I2_96 were washed in separate experiments with solutions containing either EDTA (125 mM) or NaCl/KCl (1:1, 125 mM each). The NaCl/KCl wash had very little effect on the measured Fe:Co ratio (39:1 vs. 27 ± 4 :1). The EDTA wash resulted in a factor of 2 decrease in the Co to Fe ratio. The Fe to Co ratio of the material synthesized with I2_11 did not change significantly after NaCl/KCl (1:1, 125 mM each) wash. For isolate I101 after washing the synthesized nanoparticles with NaCl/KCl (1:1, 125 mM each) the cobalt content dropped by a factor of 2 and decreased even further after a 125 mM ETDA wash. Although the above washing procedures decreased the cobalt content in the material, still there were significant amounts of cobalt present (at least 1 ng). Thus, the presence of Co$^{2+}$ as counter-cation seems unlikely, the possibility that at least some of it is coordinated more tightly to free imidazoles (or other native bases) of the modified RNA sequence can not be ruled out. However, if complexation to the RNA were the only source of Co$^{2+}$ in the samples, the Fe:Co ratio would be expected to be significantly less than that observed.

To test whether the amount of cobalt incorporated into the particles could be increased; the CoCl$_2$ precursor concentration was increased by 100 fold. As a result, approximately 3 times more Co was incorporated into the particles giving 10 ± 4 Fe : Co mole ratio in contrast with 27 ± 4 Fe : Co mole ratio found previously under standard incubation conditions. This result was in agreement with the hypothesis that cobalt ions were incorporated into the particles as opposed to simply acting as a counter-cation to RNA backbone.
Table 1.4 Iron to cobalt ratios for different isolates after different washing procedures.

<table>
<thead>
<tr>
<th>Isolate Name</th>
<th>dH₂O Fe : Co molar ratio</th>
<th>125 mM NaCl, KCl Fe : Co molar ratio</th>
<th>125 mM EDTA Fe : Co molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>12_96</td>
<td>27 (± 4) : 1</td>
<td>39 : 1</td>
<td>60 (± 21) : 1</td>
</tr>
<tr>
<td>12_11</td>
<td>32 (± 6) : 1</td>
<td>38 : 1</td>
<td>no data</td>
</tr>
<tr>
<td>1101</td>
<td>24 (± 3) : 1</td>
<td>42 : 1</td>
<td>65 : 1</td>
</tr>
<tr>
<td>12_96</td>
<td>10 (± 4) : 1</td>
<td>no data</td>
<td>no data</td>
</tr>
</tbody>
</table>

Another concern was that the iron and cobalt were forming separate particles. To determine if cobalt alone could be utilized by RNA to make particles, pure CoCl₂ (188 mM, 99.999%) was incubated with sequence I2_96 for 100 min. The TEM analysis reviled low abundance of particles 2 ± 1 nm in diameter in contrast to 8 ± 3 nm particles obtained when both iron and cobalt were present in the incubation. This observation indicated that isolate I2_96 was capable of forming smaller particles with cobalt alone. To confirm this result, cobalt only incubations were analyzed by LS counting. The amounts of RNA retained with and without the magnetic field were compared. On average the fraction of RNA bound to the iron oxide particles resuspended by means of magnetic field was 1.1 ± 0.7 %. When no magnetic field was applied 0.02 ± 0.01 % of the RNA was retained. Higher RNA recovery when partitioning the synthesized material with the magnet suggested that isolate I2_96 formed magnet responsive particles from cobalt alone. Additionally cobalt only incubations were analyzed on 6% denaturing PAGE; however no gel shift corresponding to high molecular weight material was observed. It was possible that cobalt only particles were not detected on the denaturing PAGE due to a much lower abundance of cobalt only particles. According to counting experiments, 2.6x less RNA was retained for cobalt only incubations compared to regular incubations with both iron and cobalt. Moreover TEM analysis has shown that cobalt only particles are much smaller.
Table 1.5 Raw ICP-MS data.

<table>
<thead>
<tr>
<th></th>
<th>Co [ng]</th>
<th>Co detection limit [ng]</th>
<th>Fe [ng]</th>
<th>Fe detection limit [ng]</th>
<th>Fe : Co molar ratio</th>
<th>Reaction volume [mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I2_96</td>
<td>5360</td>
<td>1</td>
<td>81000</td>
<td>30</td>
<td>16.1 : 1</td>
<td>14.5</td>
</tr>
<tr>
<td>I2_96</td>
<td>810</td>
<td>0.4</td>
<td>23400</td>
<td>4</td>
<td>32.2 : 1</td>
<td>2.9</td>
</tr>
<tr>
<td>I2_96</td>
<td>11</td>
<td>0.3</td>
<td>340</td>
<td>8</td>
<td>32.5 : 1</td>
<td>10</td>
</tr>
<tr>
<td>Salt wash</td>
<td>650</td>
<td>0.4</td>
<td>24100</td>
<td>4</td>
<td>39.2 : 1</td>
<td>5.835</td>
</tr>
<tr>
<td>I2_96</td>
<td>1.54</td>
<td>0.1</td>
<td>45</td>
<td>8</td>
<td>30.9 : 1</td>
<td>0.555</td>
</tr>
<tr>
<td>EDTA wash</td>
<td>3.3</td>
<td>0.4</td>
<td>280</td>
<td>10</td>
<td>89.5 : 1</td>
<td>10</td>
</tr>
<tr>
<td>I2_96</td>
<td>1.04</td>
<td>0.09</td>
<td>15</td>
<td>8</td>
<td>15.4 : 1</td>
<td>5</td>
</tr>
<tr>
<td>Co ex</td>
<td>5.48</td>
<td>0.09</td>
<td>20</td>
<td>8</td>
<td>3.9 : 1</td>
<td>5</td>
</tr>
<tr>
<td>I2_11</td>
<td>2810</td>
<td>0.4</td>
<td>61700</td>
<td>4</td>
<td>23.5 : 1</td>
<td>12.5</td>
</tr>
<tr>
<td>I2_11</td>
<td>4.5</td>
<td>0.4</td>
<td>170</td>
<td>8</td>
<td>39.8 : 1</td>
<td>10</td>
</tr>
<tr>
<td>Salt wash</td>
<td>1300</td>
<td>0.4</td>
<td>47200</td>
<td>4</td>
<td>38.4 : 1</td>
<td>12.5</td>
</tr>
<tr>
<td>I101</td>
<td>3100</td>
<td>0.4</td>
<td>57700</td>
<td>4</td>
<td>19.9 : 1</td>
<td>12.5</td>
</tr>
<tr>
<td>I101</td>
<td>20</td>
<td>0.3</td>
<td>550</td>
<td>8</td>
<td>29.0 : 1</td>
<td>10</td>
</tr>
<tr>
<td>salt wash</td>
<td>1.43</td>
<td>0.4</td>
<td>57300</td>
<td>4</td>
<td>42.2 : 1</td>
<td>12.5</td>
</tr>
<tr>
<td>I101</td>
<td>7.9</td>
<td>0.4</td>
<td>490</td>
<td>10</td>
<td>65.5 : 1</td>
<td>10</td>
</tr>
</tbody>
</table>
The cobalt only experiments indicated that isolate I2_96 could mediate the formation of 2 nm nanoparticles from cobalt alone. However when iron was present in reaction mixture after 100 minutes incubation 2 nm particles were not detected. That suggested that cobalt doped iron oxide as opposed to separate iron and cobalt species were being synthesized. However, there is not enough evidence to support a definitive conclusion.

In order to prove that RNA was involved in the nanoparticle synthesis a no RNA control ICP-MS experiments were performed. Iron and cobalt precursors were incubated following previously described procedure except no RNA was present. After 100 minutes incubation the solid material was separated on 100 kDA molecular weight cut off filter followed by magnet partitioning. For best results incubations were scaled up to 10 mL and repeated seven times. Average iron and cobalt content detected by ICP-MS was $4 \pm 3$ ng and $0.5 \pm 0.3$ ng respectively. The amounts of iron and cobalt detected for the nanoparticles synthesized with RNA, for the same reaction volume, were significantly larger; $305 \pm 100$ ng for iron and $8 \pm 6$ ng for cobalt. The variability of the results was large, most likely due to low efficiency and pure reproducibility of magnetic partitioning, above results confirm our previous observations that RNA is responsible for nanoparticle synthesis.

To prove that the material synthesized by isolate I2_96 interacted with the magnetic field a series of experiments were performed where the amount of RNA retained with and without a magnetic field were compared. On average the fraction of RNA bound to the iron oxide particles resuspended by means of magnetic field was $2.9 \pm 0.3 \%$. When no magnetic field was applied only $0.3 \pm 0.2 \%$ of the RNA was retained. Applying a magnetic field increased RNA retention almost by an order of magnitude. Moreover when analogues experiment was done using the random imidazolyl-modified pool, fraction of the RNA bound to the iron oxide particles resuspended with the magnet was only $0.2 \pm 0.03\%$. When no magnetic field was applied $0.2 \pm 0.1\%$ of the RNA was retained. Starting random RNA pool did not show any difference between the amount of the RNA recovered with and without the magnet. In contrast when using isolate I2_96 the amount of the RNA retained using magnetic field was an order of magnitude larger compared to no magnet partitioning. These experiments confirmed the sequence dependence of the magnetic iron oxide formation phenomenon.
The results from magnet versus no magnet partitioning experiments indicate that isolate I2_96 synthesized material that responded to magnetic field. Among possible iron oxide species magnetite (Fe₃O₄) and maghemite (γ-Fe₂O₃) are magnetic, therefore it could be concluded that either one or a combination of those were being synthesized.

1.1.3 RNA studies

During the selection we introduced an imidazolyl modification into the RNA in order to provide additional metal coordination sites and proton shuttling mechanisms beyond those provided by the heterocyclic nitrogens present in native RNA. The following section discusses the importance of the imidazol modification. Moreover truncation experiments were also performed to determine how many bases of the RNA sequence were required to maintain activity.

To test whether the imidazolyl groups were essential for nanoparticle synthesis, the isolate I2_96 was transcribed using native UTP and incubated with iron and cobalt precursors, FeCl₂, CoCl₂ at 150 µM and 37.5 µM respectively and KCl, NaCl 100mM and HEPES buffer 50mM. The synthesized material was partitioned by means of magnetic field and as a comparison no magnet partitioning was performed. The material resuspended from the partitioning procedure was analyzed by LS counting for recovered RNA. The amounts of RNA retained with and without a magnetic field were compared. On average the fraction of RNA recovered using magnetic field was 0.06 ± 0.04 %. When no magnetic field was applied 0.04 ± 0.03 % of the RNA was retained. Statistically there was no difference between those data, which suggests that the imidazolyl modification was required to mediate the formation of material that responded to magnetic field.

To evaluate the effect of I2_96 truncation on its activity three truncated RNA sequences were derived from isolate I2_96 via polymerase chain reaction. The following truncated isolates were obtained:

**I2_96 T1** 63-mer with 3’ primer binding sequence removed from the 3’ end:
5’5P8CCAUUGACAUACAACAAUUUAAUACAUCCUUCUUCGAUCCCA 3’

**I2_96 T2** with 15 bases removed from the 3’ end giving 72 base long RNA sequence:
5’5P8CCAUUGACAUACAACAAUUUAAUACAUCCUUCUUCGAUCCAUUCGACAGG 3’

**I2_96 T3** a 33-mer, made by removing 54 bases downstream from conserved region:
The truncates were incubated with iron and cobalt precursors according to the previously described protocol for 100 minutes. The resulting material was separated by magnetic partitioning and imaged with TEM. Truncate I2_96 T2 (72-mer) produced nanoparticles 2.2 ± 0.3 nm in size. Truncate I2_96 T1 (63-mer) formed 2.6 ± 0.2 nm sized particles and the shortest truncate I2_96 T3 (33-mer) produced nanoparticles 3 ± 0.3 nm in size. Additionally to confirm the formation of nanoparticles incubation with each truncate was analyzed by PAGE in denaturing conditions. Figure 1.10 shows a gel image containing the transcription product lane for each truncate (lanes 1, 4 and 7), each truncate incubated with iron and cobalt precursors (lanes 2, 5 and 8) and truncates incubated without metal precursors (lanes 3, 6 and 9). As expected there was a gel shift corresponding to I2_96 truncates bound to high molecular weight material (lanes 2, 5 and 8). When no metal precursors were present in the reaction no gel shift was detected (lanes 3, 6 and 9).
Finally LS counting experiments were performed to further test the activity of the truncated I2_96 RNA. The amounts of RNA recovered when partitioning with and without the magnet were compared for each truncate and they are listed in Table 1.6. The best results for RNA recovery for magnet versus no magnet partitioned incubations was obtained for the longest truncate T2. When applying magnet partitioning, an order of the magnitude more RNA was recovered in contrast to washing without the magnet. The shortest truncate T3 had the highest RNA retention when partitioned with magnetic field, however the variability if these data was also the largest. The smallest RNA recovery when partitioning with the magnet was observed for truncate T1. Compared to full length isolate I2_96 the RNA recovery for the truncated sequences using magnetic field was significantly lower indicating that shortening the RNA sequence affected the activity.

Table 1.6 Fraction of RNA recovered during partitioning with and without the magnet for isolate I2_96 truncates

<table>
<thead>
<tr>
<th></th>
<th>Magnetic Field Partitioned [% RNA recovery]</th>
<th>Magnetic Field Partitioned [% RNA recovery]</th>
</tr>
</thead>
<tbody>
<tr>
<td>I2_96 T1</td>
<td>0.09 ± 0.06</td>
<td>0.026 ± 0.005</td>
</tr>
<tr>
<td>I2_96 T2</td>
<td>0.3 ± 0.2</td>
<td>0.028 ± 0.005</td>
</tr>
<tr>
<td>I2_96 T3</td>
<td>0.9 ± 0.7</td>
<td>0.3 ± 0.3</td>
</tr>
</tbody>
</table>

The above results from TEM, PAGE and LS counting, suggested that despite partial sequence truncation of isolate I2_96 it still retained its activity in mediating the formation of iron oxide nanoparticles. However the synthesized nanoparticles were smaller as determined by TEM and less material was being synthesized as shown lower RNA recovery by LS counting experiments. This suggested that partial removal of the I2_96 sequence must have compromised its secondary structure thereby affecting the activity of the truncated RNA.
1.2 Summary

We have shown that RNA sequences can be selected based on a materials property. Magnetic partitioning of a large (ca. $10^{14}$) random sequence RNA library resulted in RNA species that mediate the formation of magnetic iron oxide nanoparticles. Selected RNA sequences were identified via standard cloning and sequencing procedures and genomically aligned giving two families based on the presence of high sequence similarity regions. Among identified sequences the conserved regions commonly contained point mutations.

Series of experiments (TEM, ICP-MS) have shown that RNA was required for the material synthesis. Moreover the sequence dependence was shown when the results from TEM imaging and RNA recovery monitoring by LS counting were compared for isolate I2_96 and the starting random RNA pool. Further studies revealed that imidazol modification was critical for I2_96 activity. The isolate I2_96 truncation experiments have showed that isolate I2_96 with 15, 24 or 54 bases removed from its 3’ end still retained its activity in mediating the formation of magnetic iron oxide nanoparticles. However the synthesized nanoparticles were smaller and the overall yield of the product was lower than for the full length sequence.

TEM studies of the material synthesized by four chosen isolates (I47, I101, I2_4 and I2_96) revealed spherical nanoparticles. TEM analysis of particles synthesized using sequence I2_96 showed that the particles grew to a diameter of $2.9 \text{ nm} \pm 0.9 \text{ nm}$ in 1 minute, and reached a diameter of $8 \pm 3 \text{ nm}$ after 100 minutes (Figure 1.7).

Material characterization attempts provided only partial information about synthesized nanoparticle composition. Various analysis attempts revealed the presence of iron, cobalt and oxygen. The relative molar ratio of iron to cobalt was determined to be about 28:1. Cobalt only experiments showed that RNA was capable of forming smaller nanoparticles with cobalt alone. Measurements of RNA retained when the particles were washed with and without the magnetic field implied that synthesized material was magnetic. At this point the only conclusion than could be made about the composition of synthesized material was that it was magnetic iron oxide magnetite ($\text{Fe}_3\text{O}_4$) or maghemite ($\text{Fe}_2\text{O}_3$) doped with cobalt, or a mixture of those.
In order to unambiguously determine the composition of synthesized nanoparticles much larger quantities of the material were required. One way of accomplishing that would be a synthesis of mg amounts of RNA and scaling up the nanoparticle synthesis. However in \textit{vitro} RNA synthesis in large quantities is very costly. Therefore we decided to utilize \textit{E. Coli} to produce the particles. It has to be noted that imidazolyl modification is not possible \textit{in vivo}, therefore a native RNA sequence capable of mediating the formation of the magnetic nanoparticles was needed. In the next chapters I will describe the selection of a native sequence capable of the magnetic nanoparticle formation and its insertion into a bacterium for particle scale up.
1.3 Experimental Section

Reagents.
All reagents were used without further purification. Milli-Q water was treated with diethylpyrocarbonate (depc) prior to use to ensure nuclease- and protease-free water.

PCR amplification of DNA Templates.
5′-primer (5′-TAATACGACTCAC-TATAGGGAGACAAGAATAAACGCTCAA-3′) and 3′-primer (5′-GCCTGTTGT-GAGCCTCCTGTCGAA-3′) were purchased from Midland Certified, Inc.. 2 nM (ss)dsDNA template were combined with 1× Taq DNA Polymerase buffer (New England Biolabs, 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl, pH 8.8, 2 mM MgSO4, 0.1% Triton X-100), 0.12 mM each of dATP, dCTP, dGTP, and dTTP, 1 µM each of 5′-primer and 3′-primer, and 0.1 U/µL Taq DNA Polymerase (New England Biolabs). PCR was performed using the following reaction parameters: 95 °C, 2 minutes; followed by multiple cycles of 95 °C, 30 seconds, 60 °C, 30 seconds, 72 °C, 45 seconds; hold at 4 °C. The dsDNA was purified using QIAquick PCR Purification Kit (Qiagen) and quantitated via UV-Vis spectroscopy.

Generation of RNA Isolates.
RNA isolates were prepared by transcription of dsDNA templates. 5× T7 RNA Polymerase buffer (homemade or from Promega) (4% (w/v) PEG 8000, 40 mM Tris-HCl, pH 8.0, 12 mM MgCl2, 5 mM dithiothreitol (DTT), 1 mM spermidine HCl, 0.002% Triton X-100), 0.2 mM each of ATP, CTP, GTP, and 5-(4-imidizolylmethyl)-UTP (*UTP), 250 nM dsDNA template, 125 nM T7 RNA Polymerase (Promega), 0.8 U/µL RNase inhibitor (Promega) were incubated at 37 °C for 6 hours to yield imidazolyl modified random pool of RNA transcripts, 87 bases long: 5′-GGGAGACAAGAATAAACGCTCAA-[40N]-TTTGACAGGAGGCTCAGAACAAGGC-3′. The [α-32P]-ATP body-labeled RNA pool was generated using an identical protocol with the addition of 30 µCi of [α-32P]-ATP per reaction. Size-exclusion membranes (Microcon 10, 10-kD cutoff) were used to separate the full-length RNA from the reaction buffer and any unincorporated NTPs. The reaction mixture was first concentrated onto the membranes by centrifugation and washed four times with either buffer containing NaCl (1 mM), KCl (1 mM), and Na3PO4 (1 mM, pH 7.2) or
RNase free water. The purified RNA was recovered from the membranes by resuspension in 50-100 µL of water. Non-radiolabeled RNA was quantitated by UV-Vis spectroscopy while radiolabeled RNA was quantitated by liquid scintillation counting using a Beckman Coulter LS-6500. Due to a presence of high molecular weight bands accompanying the band of interest obtained the RNA had to be gel purified during each round of the selection. To do so RNA was applied to a 6% denaturing PAGE, the correct size band (with the same mobility as the starting random RNA pool) was excised and electroeluted at a constant 2 W per sample for 3 hours.

**Formation of Nanoparticles.**
RNA sequences (0.9 µM) were incubated in the presence of 150 µM FeCl₃ (Acros), 37.5 µM CoCl₂ (Riedel-de Hasen), 100 mM NaCl (Riedel-de Hasen), 100 mM KCl (Riedel-de Hasen), 50 mM HEPES pH 7.3 (Fisher BioReagents). Iron and cobalt salts were dissolved in RNase free Mili-Q water to give a concentration of 1 mM and 0.5 mM respectively, followed by degassing with Ar for at least 15 minutes to remove oxygen. The incubations were performed in aqueous solution for 5 hours at ambient temperature. To eliminate any RNA bound to the sides of the eppendorf tube the reaction mixture was transferred to a new tube. Next RNA molecules that synthesized magnetic nanoparticles and remained bound to them, were separated from remaining inactive RNA and unused reagents using magnetic field partitioning. A tube containing incubated material was placed on a permanent magnet (ceramic coated neodymium, 35 mm x 25 mm x 5 mm, N35) for 12 hours. Magnetic nanoparticles with bound RNA were attracted by the magnet and remained in the tube while the solution was completely removed. The particles that remained in the tube were then washed 4 times in the presence of the magnet with 200 µL of 1x buffer (K⁺, Na⁺, PO₄³⁻) and resuspended in 100 µL of dH₂O

**Reverse Transcription.**
RNA retained from magnet partitioning was combined with 0.5× 1st Strand Buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM MgCl₂), 0.5 mM each of dATP, dCTP, dGTP and dTTP, 2 µM 3’ primer, and 0.4 U/µL AMV Reverse Transcriptase (New England Biolabs). The RNA was reverse transcribed at 42 °C for 45 minutes. Following reverse transcription,
the enzyme was inactivated by heating at 72 °C for 15 minutes. The cDNA was brought forward into amplification without further purification.

**Dideoxy-Mediated Bulk Sequencing of Evolved Pools.**

**End-labeling 5’-primer:** Twenty picomoles of 5’-primer were end-labeled with 1 µM \( \gamma \)-32P-ATP in the presence of 0.6 U/µL of bacteriophage T4 Polynucleotide Kinase (New England Biolabs). The reaction mixture was incubated at 37 °C for 30 minutes. Following the reaction, the polynucleotide kinase was inactivated by heating at 90 °C for 2 minutes. A 250 µL aliquot of water was added to the labeling mixture and was concentrated onto a 10-kD molecular size cutoff filter to remove kinase buffer and unincorporated phosphate. The end-labeled primer (*5-primer) was then recovered from the membrane by resuspension in 25-50 µL of water.

**Dideoxy Mediated Cycle Sequencing:** The ddNTP/dNTP terminator mixes were prepared using the following ratios: dNTP/ddATP (1:60), dNTP/ddCTP (1:40), dNTP/ddGTP (1:20) dNTP/ddTTP (1:80). A 2 µL aliquot of the appropriate terminator mixture was added to a 4 µL aliquot containing the DNA template, 0.1 U/µL *Taq* DNA Polymerase, 1 µM *5- primer, and 1× sequencing buffer. Therefore, for one sample, there were four terminator/reaction mixtures, each corresponding to the four deoxyribonucleotides. The sequencing reaction had undergone 30 cycles of PCR (95 °C, 2 min; 95 °C, 30 sec; 60 °C, 30 sec; 70 °C, 30 sec; repeat for 30 cycles; hold at 4 °C). A 6 µL aliquot of each reaction was then loaded onto a preheated 10 % Native PAGE (2000 V, 50 mA, constant 30 W, ~ 3.5 h). The gel was imaged using a Packard Cyclone Storage Phosphor System and Packard OptiQuant Image Analysis Software.

**Cloning of Evolved Pools.**

**Evolved pool preparation:** In preparation of cloning the dsDNA copy of evolved pool, the sample was amplified using standard PCR conditions (see “Amplification of DNA Templates”), incorporating a custom 5’-primer not previously used in the selection amplifications. The 5’ Cloning Primer (5CP, 5- GGGAGACAAGAATAAACGCTCAA-3), was synthesized by Midland Certified, Inc. The amplification product was by 6% PAGE purification. The ds5CP-DNA product was excised from the gel and electroeluted at a
constant 10 W for 4 hours. Following electrophoresis, the ds5CP-DNA was purified using QIAquick PCR Purification Kit (Qiagen). The resulting ds5CP-DNA was quantitated by UV-Vis spectroscopy.

**Polishing dsDNA templates:** Following the instructions provided with the PCR-Script™ Amp Cloning Kit (Stratagene) the ds5CP-DNA sample was polished using cloned 0.04 U/µL *Pfu* DNA Polymerase, 1× polishing buffer and 0.19 mM each of dATP, dCTP, dGTP, and dTTP. The polishing reaction was mixed gently and then incubated at 72 °C for 30 minutes. *Pfu* DNA polymerase generated high fidelity, blunt-ended PCR products (two complementary strands of DNA are of equal length, without ssDNA overhangs).

**Ligating the Insert to Vector:** Blunt-ended ligation was performed by incubating 0.3 µM polished 5CP-DNA with the predigested 1 ng/µL pPCR-Script™ Amp SK(+) cloning vector (ca. 30 kDa) (Stratagene), 1× reaction buffer, 0.5 mM rATP, 0.5 U/µL *Srf I* restriction enzyme, 0.4 U/µL T4 DNA Ligase, and water to a final volume of 10 µL. Using the restriction enzyme in the ligation procedure maintained a high concentration of digested vector and increased the recombination (insert incorporation) yield. The ligation reaction was mixed gently and then incubated at 25 ºC for 2 hours. Following the incubation, the ligase was inactivated by heating at 65 °C for 10 minutes. *Srf I* is a novel rare-cleavage restriction enzyme that recognizes the oligonucleotide sequence 5’-GCCCGGGC-3’. Prior to ligation the evolved pool was tested for *Srf I* restriction site and no digest was detected.

**Transformations:** The XL10-Gold Kan® Ultracompetent cells (Stratagene) were thawed on ice and gently mixed by tapping. A 40 µL aliquot of cells was added to a chilled 10-mL falcon tube and a 1.6 µL aliquot of XL10-Gold β-mercaptoethanol mix (Stratagene) was added to the cells. The cells were swirled gently and incubated on ice for 10 minutes, with swirling every 2 minutes. A 2 µL aliquot of the ligation product was then added to the cells. The cells were incubated on ice for an additional 30 minutes. Following this incubation, the cells were heat pulsed in a 42 ºC water bath for 30 seconds (the temperature and length of heat pulse was critical for obtaining the highest efficiencies). Following the heat pulse, the cells were placed on ice for 2 minutes; then 0.45 mL of preheated (42 ºC) NZY+ broth was added to the cells and incubated at 37 ºC for 1 hour with shaking at 250 rpm. A sterile spreader was used to plate the transformed cells onto already prepared LB Amp agar (Fermentas) plates containing ampicillin 50 µg/mL, and for blue and white color screening
IPTG 100 µg/mL and X-Gal. The plates were incubated at 37 °C for 15-18 hours. To insure that obtained white colonies do not contain multiple inserts, individual white colonies were picked with sterile toothpicks and streaked onto prepared LB Amp agar plates containing IPTG and X-Gal. These plates were then incubated for 15-18 hours at 37 °C. Following this incubation, the plates were placed at 4 °C to enhance the white color of the colonies.

In transformation procedure transformed cells were plated on LB Ampicilin agar containing IPTG and X-Gal (for blue and white color screening, explained below). The pPCR-Script™ Amp SK(+) cloning vector contained the ampicillin resistance gene, and the lacZ’ gene that codes for part of the enzyme β-galactosidase. Cloning with pPCR-Script Amp SK(+) involved insertional inactivation of the lacZ’. The recombinants were identified based on their inability to synthesize β-galactosidase. When β-galactosidase was synthesized X-gal was broken down to a deep blue colored product. When X-gal (plus an inducer of the enzyme isopropylthiogalactoside, IPTG) was added to the agar, along with ampicillin, then non-recombinant colonies were colored blue, whereas recombinants with a disrupted lacZ’ gene were white. Non-transformed colonies did not grow at all since they lacked the antibiotic resistance.

Sequencing of Evolved Pools.

Inoculation of Colonies: Following the 37 °C incubation of transformations plated on LB Amp Agar containing IPTG and X-Gal, individual white colonies were picked using a sterile toothpick and loaded into a 96-well inoculating plate (plate 1) containing 0.90 mL liquid LB Amp Agar (Fermentas). The samples were incubated overnight (16-18 h) at 37 °C with shaking at 222 rpm, to generate more bacterial mass. Following this first overnight inoculation, a 5 µL aliquot of inoculate was transferred to a second inoculating plate containing fresh liquid LB Amp agar for further cell enrichment. The second inoculation (plate 2) was incubated at 37 °C with shaking at 222 rpm overnight (16-18 h) while plate 1 was stored at 4 °C. Following the second set of inoculations, pellet of cells in plate 2 were formed by centrifuging the inoculation plate at 2700 rpm for 15 minutes. The liquid media was poured from the sample wells and the sample plate was inverted and gently tapped to remove excess media with careful attention not to loosen and dump the pellets. The plate 1
inoculations were transferred to plate 2, plate 2 was centrifuged once more to concentrate and
pellet the cells, and the liquid media was removed.

**Alkaline Lysis of Cells:** The bacterial alkaline lysates were prepared using the Bio Robot
9600 (Qiagen) courtesy of the Genome Research Laboratory (GRL), Centennial Campus, NC
State University. The following protocol was performed: Each bacterial pellet was
resuspended in 0.3 mL Buffer R1 containing RNase I enzyme and mixed by vortexing the
96-well sample block for 10 minutes. A 0.3 mL aliquot of Buffer R2 was added to each well
and the block was vortexed for 2 minutes and incubated at room temperature for 5 minutes.
Following the room temperature incubation, a 0.3 mL aliquot of Buffer R3 was added to each
well and the block was vortexed for an additional 2 minutes. A QIAfilter 96-well plate
(Qiagen) was placed in position on the QIAvac 96 manifold while a collection block was
placed into the base of the manifold. The lysates were then transferred from the sample block
to the wells of the QIAfilter 96-well plate. Vacuum was applied (-200 to -300 mbar) until the
lysates were completely transferred to the collection block in the base of the manifold. To
desalt and concentrate the dsDNA, 0.7-volumes of room temperature isopropanol was added
to each well, the block was taped and mixed immediately by inverting three times. The
collection block was centrifuged at 2800 rpm for 15 minutes at room temperature to pellet
the plasmid DNA. The supernatant was removed by quickly inverting the collection block
followed by tapping to remove residual supernatant. Each pellet was then washed with 0.5
mL of 70% ethanol and centrifuged at 2800 rpm for 2 minutes to re-concentrate the pellets.
The wash solution was removed using the same technique mentioned previously and the
pellets were dried under vacuum. The dsDNA pellets were redissolved in 35 µL of 10 mM
Tris-Cl, pH 8.5 and quantitated by UV-Vis spectroscopy.

**BigDye Terminator Cycle Sequencing:** To each well of a 96-well PCR plate the following
material was added: 4 µL of BigDye Terminator Mix (containing MgCl2, dNTPs, enzyme
and reaction buffer), 0.32 µM of 5′ Sequencing Primer (5SP, 5*-GTAATACGACTCACTATA-GGGC-3*), 200-500 ng of dsDNA template and water to a
final volume of 10 µL. Cycle sequencing was performed using a GeneAmp 9700
thermocycler: 96 °C, 10 sec; 50 °C, 30 sec, 60 °C, 4 min, repeat for 30 cycles; hold at 4 °C.

**Purification of Cycle Sequencing:** A 96-well short plate (Edge BioSystems Performa® DTR)
was centrifuged at 1141 rpm for 8 minutes to remove storage buffer; the eluate was
discarded. The sequencing reaction samples were brought up to 20 µL with water and were
then transferred to the center of each purification column in the 96-well short plate. The short plate was then centrifuged at 1141 rpm for 5 minutes and the eluate containing the purified dsDNA product retained. The sequences were identified on an Applied Biosystems ABI 3700#1 sequencer (Genome Research Laboratory, NC State University).

**Transmission Electron Microscopy.**
Bright-field images were obtained using TEM at the University of North Carolina School of Dentistry using a Philips CM12 transmission electron microscope operating at 100 kV accelerating voltage. To prepare samples for analysis, an aqueous solution of RNA-bound particles was drop-cast onto carbon-coated copper TEM grids (formvar support, 300 mesh, Ted Pella). Bright-field images were captured digitally with Digital Micrograph using a Gatan 780 DualVison camera.

**High resolution Transmission Electron Microscopy**
High resolution electron microscopy was performed at the Shared Materials Instrumentation Facility, Duke University using a Hitachi HF-2000 FEG TEM operation at 200kV accelerating voltage. To prepare samples for analysis, an aqueous solution of RNA-bound particles was drop-cast onto carbon-coated copper TEM grids (formvar support, 300 mesh, Ted Pella).

**X-Ray Photoelectron Spectroscopy (XPS)**
XPS analysis was conducted at Analytical Instrumentation Facility, NCSU using Riber LAS-3000. Samples were analyzed by excitation of specimen with soft X-rays (source Mg anode, 1254 eV) followed by energy analysis of resulting photoelectron emission. The sample needed to be sputtered with 5 keV of Ar$^+$ ions for 10-30 min to obtain the strongest Fe and Co signals (about 40 Å of were removed from the surface eliminating possible salts and any surface contamination). To generate sufficient amount of colloidal material the incubation needed to be scaled up 200x. This required synthesizing about 3.32 mg of RNA. To generate such an excessive amount of RNA the Megascript® High Yield Transcription Kit was used (see “Transcription scale up”). To minimize increased byproduct formation, due to scale up, ten 10 mL incubation reactions were conducted for 2h at ambient temperature. The obtained
solid material was separated from unused reactants and washed 3x with dionized water on 50K molecular weight cut off filters (Centricon 50), followed by magnetic material separation on macroscopic magnet. XPS samples were prepared by consequently evaporating small drops of magnetic material, resuspended in deionized water, onto a silica wafer.

**Inductively Coupled Plasma Mass Spectrometry (ICP-MS)**

ICP-MS analysis was performed by West Coast Analytical Service facility. For best results incubation reactions were scaled up at least 5x and the solid material was separated on 100 kDa molecular weight cut off filters, washed four times with dH₂O and resuspended, followed by magnetic separation.

**Preparation of truncated isolate I2_96**

A series of 22 bases long ssDNA anti sense oligonucleotides (primers) complementary to the different portions of isolate I2_96 were designed as shown and purchased from Midland Certified, Inc.:

3P8T1 5' CCTGTCAATGGAATCGAAGAAGG 3'
3P8T2 5' TGG AATCGAAGAAGGATGTATAAT 3'
3P8T3 5' ATGTCAATGGTTGAGCGTTTATTC 3'

The truncated DNA templates of isolate I2_96 were generated by PCR, (see “Amplification of DNA Templates”). The primers were designed to hybridize to the DNA template upstream from the typical 3P8 binding site. Resulting PCR products were purified on 6% native polyacrylamide gel electrophoresis (PAGE) and then used as templates for in vitro transcription.
1.4 References


CHAPTER 2: SELECTION OF NATIVE RNA MOLECULES MEDIATING THE GROWTH OF MAGNETIC IRON OXIDE NANOPARTICLES

In Chapter 1, a new RNA in vitro selection method was described in which RNA sequences capable of mediating the formation of magnetic cobalt iron oxide nanoparticles were isolated from a large random sequences RNA pool. In that work, the RNA contained imidizolyl-modified uridine, which was hypothesized to aid in metal ion coordination and general acid/base catalysis. To determine whether the modification was required for mediating the synthesis of magnetic cobalt iron oxide nanoparticles, an analogous selection was conducted using a native RNA random pool. It will be shown that native RNA sequences that mediate the formation of cobalt iron oxide nanoparticles could in fact be isolated with important consequences. It will be shown in Chapter 3 that native RNA sequence which codes for iron oxide nanoparticles could be genetically encoded into bacteria to create living magnetic nanoparticle factories.

2.1 Results and discussion

2.1.1 Selection process

The selection followed the cycle analogues to imidazolyl modified selection, shown in Scheme 1, section 1.1.1. The same chemically synthesized (ABI 391) library was used, containing ~10^{14} unique ssDNA sequences, 87 bp in length, containing a center region of 40 bp, random in sequence, flanked by regions of defined sequence, at the 5’ and 3’ ends. In step 1 T7 RNA polymerase was used to transcribe the dsDNA library into an ssRNA library containing ca. 10^{14} sequences. During step 1 transcription was carried out following the procedure described previously with the exception of native UTP use. The RNA library obtained was purified on a 10K molecular weight cut-off filter by washing four times with 1x buffer (Na^{+}, K^{+}, PO_4^{2-}) and resuspended in deionized, RNase free water. In step 2, the RNA library (900 nM) was incubated with the metal salts FeCl_{2} and CoCl_{2} at 150 µM and 37.5 µM respectively, additionally KCl, NaCl 100mM and HEPES buffer 50mM were present. Prior to incubation, argon gas was bubbled through iron and cobalt chloride solutions for 15 min to remove oxygen and therefore prevent metals from oxidizing. The incubation was performed
in aqueous solution for 5 hours at ambient temperature. RNA molecules, that synthesized magnetic nanoparticles and remained bound to them, were separated from remaining inactive RNA and unused reagents using magnet partitioning. A tube containing incubated material was placed on a macroscopic magnet for 12h. Magnetic nanoparticles with bound RNA were attracted by the magnet and remained in the tube while the solution was removed; the particles were washed 4 times with 200 µL of 1x buffer (K⁺, Na⁺, PO₄³⁻) and resuspended in 100 µL of dH₂O. To make sure that the RNA sequences that non-specifically bound to the sides of the tube were not carried forward, a counter selection step was introduced. Prior to magnetic partitioning the reaction mixture was transferred to a fresh tube, thus eliminating any RNA bound to the tube. The selected RNA was reverse transcribed to give a cDNA copy of the “winning” RNA sequences. The obtained cDNA was amplified, without purification, by means of PCR. PCR amplification completed the selection cycle and provided a dsDNA template enriched in the winning sequences and ready for T7 RNA polymerase transcription at the beginning of the next cycle.

Selection progress was monitored by recording the amount of RNA recovered every cycle due to binding to magnetic particles. Figure 2.1 shows how the RNA recovery changed as a function of the cycle number. The fraction of retained RNA through out the selection fluctuated. The highest recovery was obtained in round 4 (0.5%) however it was unlikely that the selection was completed at that round. The selection was carried through round 8 in order to compare the results with the imidazolyl modified selection described in Chapter 1.

To estimate the efficiency of the magnetic field partitioning step and to ensure that the RNA was not retained in the test tube due to binding to the tube walls, control incubations were carried out in rounds 5 through 8. The control incubations were partitioned without the presence of magnetic field. The tube containing the reaction mixture was washed 4 times with 200 µL of 1x buffer (K⁺, Na⁺, PO₄³⁻) and resuspended in 100 µL of dH₂O. An aliquot of the resuspended material was counted on the LS and compared with the results obtained from

![Figure 2.1 Selection monitoring. Fraction of RNA retained in partitioning step as a function of a selection cycle number.](image_url)
magnet-partitioned incubations, Table 2.1. As the selection progressed after the initial drop in round 6, the percentage of the input RNA recovery significantly increased in rounds 7 and 8 for the sample partitioned using a magnetic field. For the incubations partitioned without the magnet the recovery in rounds 5 through 7 was only 0.005% on average. In round 8 however the recovery increased to 0.11%. A slight increase in recovery percentage of the RNA in no magnet controls was most likely due to the presence of the sequences that were nonspecifically binding to the sides of the tube. However during the selection, the no magnet counts were consistently about three times lower than magnet partitioned samples, with the exception at round 6. This data supported the hypothesis that the particles with bound RNA remained in the tube, during the washing procedure, due to the particle interaction with magnetic field.

Table 2.1 Fraction of RNA recovered during magnet partitioning versus no magnet partitioning.

<table>
<thead>
<tr>
<th></th>
<th>Magnetic Field Partitioned [%recovery]</th>
<th>No Magnetic Field [%recovery]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Round 5</td>
<td>0.01 ± 0.003</td>
<td>0.003 ± 0.003</td>
</tr>
<tr>
<td>Round 6</td>
<td>0.004 ± 0.002</td>
<td>0.005 ± 0.002</td>
</tr>
<tr>
<td>Round 7</td>
<td>0.048 ± 0.004</td>
<td>0.006 ± 0.003</td>
</tr>
<tr>
<td>Round 8</td>
<td>0.29 ± 0.02</td>
<td>0.11 ± 0.02</td>
</tr>
</tbody>
</table>

Control experiments in which Co$^{2+}$ and Fe$^{2+}$ were incubated alone or in solutions containing heterocyclic nitrogen such as histidine monomer or histidine rich polypeptide CGWPE(H)$_{10}$ under identical reaction and magnetic field partitioning conditions yielded no nanoparticles as observed by TEM. Those experiments helped to rule out the possibility that the nanoparticles formed spontaneously.
Additionally, the selection was monitored by gel shift analysis. Aliquots of the material synthesized in rounds 6 through 8 were analyzed on 6% denaturing PAGE. Figure 2.2 shows gel image from round 7, in which lanes 1 through 4 correspond to pure transcript, incubation solution partitioned without the magnet, magnet partitioned incubation and non-partitioned incubation solution, respectively. In lane 4 there is a pronounced gel shift corresponding to the radioactively labeled RNA bound to high molecular weight material. Material from this lane was not partitioned; it was loaded on the gel directly following the incubation. Lane 3 corresponds to synthesized material that was magnet partitioned. This lane also contains gel shifted, high molecular weight material. As a control material partitioned without the magnet was also analyzed onto the gel, lane 2, to rule out the possibility of retaining the material due to nonspecific binding to the sides of the tube. There was no gel shift detected when partitioning without the magnet.

Figure 2.2 Gel shift analysis of the material synthesized in round 7: Lane 1 contains pure transcript, Lane 2 contains incubation solution partitioned without the magnet, Lane 3 contains incubation solution that was magnet partitioned, Lane 4 contains material that was not partitioned.
Material synthesized by the round 8 RNA evolved pool was investigated by transmission electron microscopy and compared to the material synthesized by the starting random RNA pool. Incubating the metal ions with the initial RNA sequence pool did not yield nanoparticles. Following 8 cycles of selection and amplification, however, spherical nanoparticles were observed by TEM (Figure 2.3). The mean particle size was 7.42 nm with the dispersity of about 30%.

![Figure 2.3 TEM image and size distribution of material synthesized by native evolved pool.](image)

Combining the results from the native selection monitored via TEM, gel shifts and percent of RNA recovery, round 8 was chosen as the end of the selection. In order to spatially resolve individual RNA sequences from the evolved pool and identify their sequences, the DNA PCR product from Step 5 of the last round of the selection was used in standard plasmid cloning procedures. The obtained sequences were genomically aligned using the sequence alignment tool (Daughters of Sequence Alignment – DOSA) and grouped into families related by semi-conserved sequence regions (Figure 2.4). Cloning and sequencing led to distinguishing 45 sequences. Two families were identified. Family 1 comprised of 58% of the library population. Twenty-six isolates shared the UUUAUUAA region of high sequence similarity. Sequences A5 and A2_6 occurred twice. The other eight sequences were grouped into Family 2 based upon a common motif AAUAAAAA. The remaining eleven sequences, so called orphans, did not share any primary sequence homology with either family or with each other.

Based on the sequence analysis it could be concluded that in order for RNA to be active in magnetic nanoparticle synthesis it was not necessary for the conserved regions to maintain 100% consensus sequence identity, one to two point mutations were commonly observed. Within the UUUAUUAAA motif the bases maintained 82% identity among
sequences from Family 1, the eighth position was the most conserved (96%). Within the AAUAAA motif the bases maintained 80% identity, positions 3 and 5 were the most conserved (90%). Compared to the sequences obtained from imidazolyl-modified selection the semi-conserved regions from native selection were one base longer and maintained higher sequence identity by 2%, which possibly helped to compensate for the lack of imidazolyl modification in the RNA. Analogously to imidazolyl-modified RNA sequences isolated in Chapter 1 the regions of high sequence similarity were shifted with respect to each other in the RNA strands.

Figure 2.4 Native RNA sequences capable of catalyzing the formation of magnetic cobalt iron oxide nanoparticles. The sequences are shown grouped into families related by semi-conserved regions (conserved regions shown in color).
2.1.2 Materials characterization

Isolates A5, A2_5, A2_42, A2_46 were subsequently chosen as representative sequences, and their ability to mediate the formation of magnetic nanoparticles was investigated by TEM. Each isolate showed spherical colloid formation. Size distribution as a function of reaction time was analyzed for isolate A5. Iron and cobalt precursors were incubated with the chosen isolate in a magnetic field for 1, 10, 100 minutes; followed by magnet partitioning. The resulting nanoparticles were analysed by TEM (Figure 2.5). The average particle sizes and their standard deviations listed in Figure 2.5 were obtained based on analysis of minimum of 100 particles. The colloid formation was detected after only 1 minute of reaction. The particles formed were 2.7 nm in size with 19% size dispersity. After 10 min the mean particle size reached 7 nm and the dispersity increased to 53%. Looking at the nanoparticle histogram for 10 minute incubation it appeared that predominantly two sizes were forming; about 5 and 12 nm. After 100 minutes of reaction time, the generated material contained larger particles, average size of 12 ± 2 nm (19% dispersity). The growth mechanism analysis revealed that during 1 minute incubation the particles formed by condensation. At longer incubation times particle coalescence and possibly Ostwald ripening were most likely responsible for the colloid growth.1,2
Figure 2.5 Time dependant size distribution for isolate A5.
The material synthesized by isolates A5 and A2_46 was analyzed by ICP-MS to determine whether it contained iron and cobalt and the relative ratio of those metals. The resulting iron to cobalt ratio was 28.6 : 1 for isolate A5 and 13.4 : 1 for A2_46 (Table 2.2). To address the possibility that Co\(^{2+}\) ions were simply ion paired to the polyanionic backbone of RNA or coordinated to the imidazolyl moieties, particles prepared with RNA A2_46 were washed in separate experiments with solution containing EDTA (125 mM). The EDTA wash decreased the Co content by factor of 2. Although the above washing procedure decreased the cobalt content in the material, significant amounts of Co were still present. As discussed in section 1.1.2 the presence of Co\(^{2+}\) as counter-cation seemed unlikely and if complexation to the RNA were the only source of Co\(^{2+}\) in the samples, the Fe:Co ratio would be expected to be significantly less than that observed. Thus, the analytical data supported the formation of a material comprised of a magnetite structure doped with cobalt.

<table>
<thead>
<tr>
<th></th>
<th>Co [ng]</th>
<th>Co detection limit [ng]</th>
<th>Fe [ng]</th>
<th>Fe detection limit [ng]</th>
<th>Fe : Co molar ratio</th>
<th>Reaction volume [mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5</td>
<td>0.7</td>
<td>0.1</td>
<td>19</td>
<td>10</td>
<td>29 : 1</td>
<td>7.8</td>
</tr>
<tr>
<td>A2_46</td>
<td>2</td>
<td>0.1</td>
<td>25.4</td>
<td>1</td>
<td>13 : 1</td>
<td>1.1</td>
</tr>
<tr>
<td>A2_46 EDTA wash</td>
<td>1.37</td>
<td>0.1</td>
<td>43</td>
<td>8</td>
<td>33 : 1</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Electron energy loss spectroscopy (EELS) was employed to identify the material synthesized by RNA sequence A5. The resulting EELS spectrum taken of a single particle is shown in Figure 2.6. Iron and oxygen were detected; however the oxidation state of iron could not be determined. The cobalt peak was not recorded which was probably due to its low abundance in the material, as indicated by ICP-MS.
To prove that the material synthesized by isolate A5 interacted with the magnetic field, a series of eight experiments were performed in which the amount of RNA retained with, and without a magnetic field, were compared. On average the fraction of RNA bound to the iron oxide particles resuspended by means of magnetic field partitioning was $1.1 \pm 0.2 \%$. When no magnetic field was applied only $0.015 \pm 0.003 \%$ of the RNA was retained. Applying a magnetic field increased RNA retention by almost two orders of magnitude. Moreover when an analogous experiment was performed using the random RNA sequence library the fraction of the RNA resuspended with the magnet was $0.04 \pm 0.03\%$. When no magnetic field was applied $0.01 \pm 0.01\%$ of the random sequence RNA library was retained. The difference between material resuspended with and without the magnet was about 50 fold larger for the isolate A5 compared to the starting random pool confirming the sequence dependence of the magnetic iron oxide formation phenomenon.

The results from magnet versus no magnet partitioning experiments indicated that isolate A5 synthesized material that responded to magnetic field. Among possible iron oxide species magnetite ($\text{Fe}_3\text{O}_4$) and maghemite ($\gamma$-$\text{Fe}_2\text{O}_3$) are magnetic, therefore it could be concluded that either one or a combination of those were being synthesized.

Figure 2.6 EELS spectrum of single particle shown in insert A synthesized with isolate A5.
2.1.3 RNA studies

In Chapter 1 a selection of imidazolyl modified RNA sequences capable of mediating magnetic iron oxide nanoparticles was described. Next we have shown that following analogues in vitro selection process native RNA sequences that mediate the iron oxide synthesis can be identified. It was of interest to determine whether introducing the imidazolyl group to the sequence that evolved without that modification would affect the RNA activity. Isolate A5 was transcribed with imidazolyl-UTP and was incubated with Fe\(^{2+}\) and Co\(^{2+}\) precursors as discussed above for 100 min at ambient temperature. The obtained material was separated by magnet partitioning and visualized by TEM. No colloid formation was detected by TEM. This experiment showed that although in separate selection imidazolyl-modified sequences that mediate the material formation were identified introducing the imidazolyl group to the sequence that evolved without that modification removed the activity.

In order to further investigate the RNA sequence importance in the material synthesis sequence truncation experiments were performed. To evaluate whether the nucleotide sequence of RNA isolate A5 can be shortened while still maintaining the catalytic activity two truncated RNA isolates were derived:

**A5 T1** with 24 bases truncated at the 3’ end giving 63 base long RNA sequence:

\[
5' 5P8UCGUCACACACAAUACAAUUACUAUUCAAC3'
\]

**A5 T2** 20-mer containing the conserved region with 6 bases up and downstream of it:

\[
5' AAUACAAUUACUAUCAAG3'
\]

(5P8 stands for the fixed forward sense primer region 5’GGGAGACAAGAAUAAACGCUAA 3’).

The above truncates were incubated with magnetite precursors according to the previously described protocol for 100 minutes. The obtained material was separated by magnetic partitioning and imaged with TEM. Truncate A5T1 formed nanoparticles 2.1 ± 0.2 nm in size, Fig. 2.7. For the 20-mer truncate A5T2 nanoparticles were not detected by TEM. Additionally, to confirm the formation of nanoparticles the incubation with truncate A5T1 was analyzed by polyacrylamide gel electrophoresis in denaturing conditions. Figure 2.8 shows a gel image containing the transcript lane (lane 1), truncate incubated with iron and cobalt precursors (lane 2) and truncate incubated without metal precursors (lane 2). As
expected there was a gel shift corresponding to A5 T1 truncate bound to high molecular weight material (lane 2). When no metal precursors were present in the reaction no gel shift was detected (lane 3). The material synthesized by truncate A5T1 was found to interact with magnetic field. The nanoparticles were washed with and without the magnetic field and resuspended solutions were analyzed by LS for RNA. When partitioning with a magnet 0.2 ± 0.2% of RNA was recovered, and 0.08 ± 0.05% when partitioning without the magnet. The statistical analysis indicated that there is no difference between samples partitioned with and without the magnet. However the standard deviation for samples partitioned using magnetic field was much larger suggesting that truncate A5T1 retained some activity towards magnetic material formation. Much lower RNA recovery for the truncate compared to the full length RNA transcript suggested that the truncation decreased the yield of material formation.
The above results suggested that despite the truncation of 24 bases from isolate A5 it still retained its activity in mediating the formation of iron oxide nanoparticles. However the synthesized nanoparticles were smaller, as determined by TEM, and less material was being synthesized, as shown lower RNA recovery by LS counting experiments. This suggested that partial removal of the A5 sequence must have compromised its secondary structure thereby influencing the activity of the truncated RNA. Truncate T2 (20-mer) on the other hand did not yield nanoparticles, as shown by TEM. This result suggested that the presence of only the conserved region was not sufficient enough to mediate the formation on iron oxide material. Other parts of the A5 sequence must have been involved in secondary structure which was disrupted by the sequence truncation.

2.2 Summary

It has been shown that native RNA sequences can be selected based on a materials property in a manner analogues to the imidazolyl modified RNA selection (see Chapter 1). Magnetic partitioning of a large (ca. $10^{14}$) random sequence RNA library resulted in RNA species that mediated the formation of magnetic iron oxide nanoparticles. Selected RNA sequences were identified via standard cloning and sequencing procedures and genomically aligned giving two families based on the presence of high sequence similarity regions. Common motifs found among the native evolved pool were one base longer that those found for the imidazolyl evolved pool. Both native and modified sequences commonly contained one or two point mutations within the regions of high sequence similarity.

The material synthesis was shown to be RNA and sequence dependant. Series of experiments (TEM, ICP-MS) have shown that RNA was required for the material synthesis. Moreover the sequence dependence was shown when the results from TEM imaging and RNA recovery monitoring by LS counting; were compared for isolate A5 and the starting random RNA pool. Truncation experiments showed the sequence length dependence on the iron oxide nanoparticle synthesis. The removal of 24 bases from the 3’ end of the A5 RNA sequence decreased the yield and size of the synthesized nanoparticles. When most of the A5 sequence was removed leaving the conserved region with 6 bases on the 5’ and 3’ ends inhibited the ability of truncate T2 to synthesize the iron oxide material.
TEM studies of the material synthesized by four chosen isolates revealed spherical nanoparticles. A 1 minute incubation with isolate A5 yielded nanoparticles less than 3 nm in size with very narrow size distribution. When allowed to grow for 100 minutes the nanoparticles reached 12 nm in size (Figure 2.5).

In an attempt to characterize the synthesized material EELS was employed and confirmed the presence of iron and oxygen. However, due to limited quantity of the material the oxidation state of iron could not be determined. The relative molar ratio of iron to cobalt was determined by ICP-MS to be 21 : 1 on average. Measurements of RNA retained when the particles were washed with and without the magnetic field implied that synthesized material was magnetic. At this point the only conclusion than could be made about the composition of synthesized material was that it was magnetic iron oxide Fe$_3$O$_4$ or Fe$_2$O$_3$ doped with cobalt, or a mixture of those.

To assemble metal oxides under ambient conditions the RNA would need to facilitate the formation of metal-oxygen bonds. It was shown that both native and imidazolyl modified RNA sequences were capable of mediating the formation of iron oxide nanoparticles. In order to form metal-oxygen bonds the RNA would need a proton shuttling mechanism. In the modified RNA imidazolyl groups could act as a Lewis base to help with deprotonation of the iron hexa-aqua complexes thereby facilitating their condensation. In native RNA an analogous role could be played by uridine groups on the native UTP through utilizing the keto-enol tautomerism.

It was very important to discover that native RNA sequences were capable of mediating the formation of magnetic iron oxide nanoparticles. In Chapter 3 it will be shown how the native sequence A5 was genetically encoded into *E. Coli* in order to produce the iron oxide nanoparticles inside of the bacteria. Such bacterial iron oxide factories would allow for the material scale up and therefore enabling its characterization by more conventional techniques (powder X-ray crystallography, Superconductive Quantum Interference Device, SQUID).
2.3 Experimental Section

Reagents.
All reagents were used without further purification. Milli-Q water was treated with diethylpyrocarbonate (depc) prior to use to ensure nuclease- and protease-free water.

PCR amplification of DNA Templates.
5′-primer (5′-TAATACGACTCAC-TATAGGGAGACAAGAATAAACGCTCAA-3′) and 3′-primer (5′-GCCTGTTGT-GAGCCTCCTGTGCAA-3′) were purchased from Midland Certified, Inc.. 2 nM (ss)dsDNA template was combined with 1× Taq DNA Polymerase buffer (New England Biolabs, 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl, pH 8.8, 2 mM MgSO₄, 0.1% Triton X-100), 0.12 mM each of dATP, dCTP, dGTP, and dTTP, 1 µM each of 5′-primer and 3′-primer, and 0.1 U/µL Taq DNA Polymerase (New England Biolabs). PCR was performed using the following reaction parameters: 95 °C, 2 minutes; followed by multiple cycles of 95 °C, 30 seconds, 60 °C, 30 seconds, 72 °C, 45 seconds; hold at 4 °C. The dsDNA was purified using QIAquick PCR Purification Kit (Qiagen) and quantitated via UV-Vis spectroscopy.

Generation of RNA Isolates.
RNA isolates were prepared by transcription of dsDNA templates. 5× T7 RNA Polymerase buffer (either homemade or from Promega) (4% (w/v) PEG 8000, 40 mM Tris-HCl, pH 8.0, 12 mM MgCl₂, 5 mM diethiothreitol (DTT), 1 mM spermidine HCl, 0.002% Triton X-100), 0.2 mM each of ATP, CTP, GTP, and UTP, 250 nM dsDNA template, 125 nM T7 RNA Polymerase (Promega), 0.8 U/µL RNase inhibitor (Promega) were incubated at 37 °C for 6 hours to yield native random pool of RNA transcripts, 87 bases long: 5′-GGGAGACAAGAATAAACGCTCGG-[40N]-TTCGACAGGAGGCTCACAACAGGC-3′. The [α-32P]-ATP body-labeled RNA pool was generated using the identical protocol but with an addition of 30 µCi of [α-32P]-ATP per reaction. Size-exclusion membranes (Microcon 10, 10-kD cutoff) were used to separate the full-length RNA from the reaction buffer and any unincorporated NTPs. The reaction mixture was first concentrated onto the membranes by centrifugation and washed four times with either buffer containing NaCl (1 mM), KCl (1 mM), and Na₃PO₄ (1 mM, pH 7.2) or RNase free water. The purified RNA
was recovered from the membranes by resuspension in 50-100 µL of water. Non-radiolabeled RNA was quantitated by UV-Vis spectroscopy while radiolabeled RNA was quantitated by liquid scintillation counting using a Beckman Coulter LS-6500. Due to a presence of high molecular weight bands accompanying the band of interest obtained the RNA had to be gel purified. To do so RNA was applied to a 6% denaturing PAGE, the correct size band (with the same mobility as the starting random RNA pool) was excised and electroeluted at a constant 10 W for 3 hours.

Formation of Nanoparticles.
RNA sequences (0.9 µM) were incubated in the presence of 150 µM FeCl₂ (Acros), 37.5 µM CoCl₂ (Riedel-de Haen), 100mM NaCl (Riedel-de Haen), 100 mM KCl (Riedel-de Haen), 50 mM HEPES pH 7.3 (Fisher BioReagents). Iron and cobalt salts were dissolved in RNase free MiliQ water to give a concentration of 1 mM and 0.5 mM respectively, followed by degassing with Ar for at least 15 minutes to remove oxygen. The incubations were performed in an aqueous solution for 5 hours at ambient temperature. To eliminate any RNA bound to the sides of the eppendorf tube the reaction mixture was transferred to a new tube. Next RNA molecules that synthesized magnetic nanoparticles and remained bound to them, were separated from remaining inactive RNA and unused reagents using magnetic field partitioning. A tube containing incubated material was placed on a permanent magnet (ceramic coated neodymium, 35 mm x 25 mm x 5 mm, N35) for 12 hours. Magnetic nanoparticles with bound RNA were attracted by the magnet and remained in the tube while the solution was completely removed. The particles that remained in the tube were then washed 4 times in the presence of the magnet with 200 µL of 1x buffer (K⁺, Na⁺, PO₄³⁻) and resuspended in 100 µL of dH₂O

Reverse Transcription.
RNA retained from magnet partitioning was combined with 0.5× 1st Strand Buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM MgCl2), 0.5 mM each of dATP, dCTP, dGTP and dTTP, 2 µM 3’ primer, and 0.4 U/µL AMV Reverse Transcriptase (New England Biolabs). The RNA was reverse transcribed at 42 °C for 45 minutes. Following reverse transcription,
the enzyme was inactivated by heating at 72 °C for 15 minutes. The cDNA was brought forward into amplification without further purification.

**Cloning of Evolved Pools.**

**Evolved pool preparation:** In preparation of cloning the dsDNA copy of evolved pool, the sample was amplified using standard PCR conditions (see “Amplification of DNA Templates”), incorporating a custom 5′-primer not previously used in the selection amplifications. The 5′ Cloning Primer (5CP, 5′-GGGAGACAAAGATAAACGCTCAA-3′), was synthesized by Midland Certified, Inc. The amplification product was by 6% PAGE purification. The ds5CP-DNA product was excised from the gel and electroeluted at a constant 10 W for 4 hours. Following electroelution, the ds5CP-DNA was purified using QIAquick PCR Purification Kit (Qiagen). The resulting ds5CP-DNA was quantitated by UV-Vis spectroscopy.

**Polishing dsDNA templates:** Following the instructions provided with the PCR-Script™ Amp Cloning Kit (Stratagene) the ds5CP-DNA sample was polished using cloned 0.04 U/µL *Pfu* DNA Polymerase, 1× polishing buffer and 0.19 mM each of dATP, dCTP, dGTP, and dTTP. The polishing reaction was mixed gently and then incubated at 72 °C for 30 minutes. *Pfu* DNA polymerase generated high fidelity, blunt-ended PCR products (two complementary strands of DNA are of equal length, without ssDNA overhangs).

**Ligating the Insert to Vector:** Blunt-ended ligation was performed by incubationg 0.3 µM polished 5CP-DNA with the predigested 1 ng/µL pPCR-Script™ Amp SK(+) cloning vector (ca. 30 kDa) (Stratagene), 1× reaction buffer, 0.5 mM rATP, 0.5 U/µL *Srf I* restriction enzyme, 0.4 U/µL T4 DNA Ligase, and water to a final volume of 10 µL. Using the restriction enzyme in the ligation procedure maintained a high concentration of digested vector and increased the recombination (insert incorporation) yield. The ligation reaction was mixed gently and then incubated at 25 ºC for 2 hours. Following the incubation, the ligase was inactivated by heating at 65 °C for 10 minutes. *Srf I* is a novel rare-cleavage restriction enzyme that recognizes the oligonucleotide sequence 5′-GCC|GGG-3′. Prior to ligation the evolved pool was tested for *Srf I* restriction site and no digest was detected.

**Transformations:** The XL10-Gold Kan® Ultracompetent cells (Stratagene) were thawed on ice and gently mixed by tapping. A 40 µL aliquot of cells was added to a chilled 1.5-mL
eppendorf tube and a 1.6 μL aliquot of XL10-Gold β-mercaptoethanol mix (Stratagene) was added to the cells. The cells were swirled gently and incubated on ice for 10 minutes, with swirling every 2 minutes. A 2 μL aliquot of the ligation product was then added to the cells. The cells were incubated on ice for an additional 30 minutes. Following this incubation, the cells were heat pulsed in a 42 °C water bath for 30 seconds (the temperature and length of heat pulse was critical for obtaining the highest efficiencies). Following the heat pulse, the cells were placed on ice for 2 minutes; then 0.45 mL of preheated (42 °C) NZY+ broth was added to the cells and incubated at 37 °C for 1 hour with shaking at 250 rpm. A sterile spreader was used to plate the transformed cells onto already prepared LB Amp agar plates containing IPTG and X-Gal (Fermentas) for blue and white color screening. The plates were incubated at 37 °C for 15-18 hours. To insure that obtained white colonies do not contain multiple inserts, individual white colonies were picked with sterile toothpicks and streaked onto prepared LB Amp agar plates containing IPTG and X-Gal. These plates were then incubated for 15-18 hours at 37 °C. Following this incubation, the plates were placed at 4 °C to enhance the white color of the colonies.

In transformation procedure transformed cells were plated on LB Ampicilin agar containing IPTG and X-Gal (for blue and white color screening, explained below). The pPCR-Script™ Amp SK(+) cloning vector contained the ampicillin resistance gene, and the lacZ’ gene that codes for part of the enzyme β-galactosidase. Cloning with pPCR-Script Amp SK(+) involved insertional inactivation of the lacZ’. The recombinants were identified based on their inability to synthesize β-galactosidase. When β-galactosidase was synthesized X-gal was broken down to a deep blue colored product. When X-gal (plus an inducer of the enzyme isopropylthiogalactoside, IPTG) was added to the agar, along with ampicillin, then non-recombinant colonies were colored blue, whereas recombinants with a disrupted lacZ’ gene were white. Non-transformed colonies did not grow at all since they lacked the antibiotic resistance.

**Sequencing of Evolved Pools.**

**Inoculation of Colonies:** Following the 37 °C incubation of transformations plated on LB Amp Agar containing IPTG and X-Gal, individual white colonies were picked using a sterile toothpick and loaded into a 96-well inoculating plate (plate 1) containing 0.90 mL liquid LB
Amp Agar (Fermentas). The samples were incubated overnight (16-18 h) at 37 °C with shaking at 222 rpm, to generate more bacterial mass. Following this first overnight inoculation, a 5 μL aliquot of inoculate was transferred to a second inoculating plate containing fresh liquid LB Amp agar for further cell enrichment. The second inoculation (plate 2) was incubated at 37 °C with shaking at 222 rpm overnight (16-18 h) while plate 1 was stored at 4 °C. Following the second set of inoculations, pellet of cells in plate 2 were formed by centrifuging the inoculation plate at 2700 rpm for 15 minutes. The liquid media was poured from the sample wells and the sample plate was inverted and gently tapped to remove excess media with careful attention not to loosen and dump the pellets. The plate 1 inoculations were transferred to plate 2, plate 2 was centrifuged once more to concentrate and pellet the cells, and the liquid media was removed.

Alkaline Lysis of Cells: The bacterial alkaline lysates were prepared using the Bio Robot 9600 (Qiagen) courtesy of the Genome Research Laboratory (GRL), Centennial Campus, NC State University. The following protocol was performed: Each bacterial pellet was resuspended in 0.3 mL Buffer R1 containing RNase I enzyme and mixed by vortexing the 96-well sample block for 10 minutes. A 0.3 mL aliquot of Buffer R2 was added to each well and the block was vortexed for 2 minutes and incubated at room temperature for 5 minutes. Following the room temperature incubation, a 0.3 mL aliquot of Buffer R3 was added to each well and the block was vortexed for an additional 2 minutes. A QIAfilter 96-well plate (Qiagen) was placed in position on the QIAvac 96 manifold while a collection block was placed into the base of the manifold. The lysates were then transferred from the sample block to the wells of the QIAfilter 96-well plate. Vacuum was applied (~200 to ~300 mbar) until the lysates were completely transferred to the collection block in the base of the manifold. To desalt and concentrate the dsDNA, 0.7-volumes of room temperature isopropanol was added to each well, the block was taped and mixed immediately by inverting three times. The collection block was centrifuged at 2800 rpm for 15 minutes at room temperature to pellet the plasmid DNA. The supernatant was removed by quickly inverting the collection block followed by tapping to remove residual supernatant. Each pellet was then washed with 0.5 mL of 70% ethanol and centrifuged at 2800 rpm for 2 minutes to re-concentrate the pellets. The wash solution was removed using the same technique mentioned previously and the pellets were dried under vacuum. The dsDNA pellets were redissolved in 35 μL of 10 mM Tris-Cl, pH 8.5 and quantitated by UV-Vis spectroscopy.
**BigDye Terminator Cycle Sequencing:** To each well of a 96-well PCR plate the following material was added: 4 µL of BigDye Terminator Mix (containing MgCl2, dNTPs, enzyme and reaction buffer), 0.32 µM of 5’ Sequencing Primer (5SP, 5'-GTAATACGACTCACTATAGGGC-3'), 200-500 ng of dsDNA template and water to a final volume of 10 µL. Cycle sequencing was performed using a GeneAmp 9700 thermocycler: 96 °C, 10 sec; 50 °C, 30 sec, 60 °C, 4 min, repeat for 30 cycles; hold at 4 °C.

**Purification of Cycle Sequencing:** A 96-well short plate (Edge BioSystems Performa® DTR) was centrifuged at 1141 rpm for 8 minutes to remove storage buffer; the eluate was discarded. The sequencing reaction samples were brought up to 20 µL with water and were then transferred to the center of each purification column in the 96-well short plate. The short plate was then centrifuged at 1141 rpm for 5 minutes and the eluate containing the purified dsDNA product retained. The sequences were identified on an Applied Biosystems ABI 3700#1 sequencer (Genome Research Laboratory, NC State University).

**Transmission Electron Microscopy.**
Bright-field images were obtained using TEM at the University of North Carolina School of Dentistry using a Philips CM12 transmission electron microscope operating at 100 kV accelerating voltage. To prepare samples for analysis, an aqueous solution of RNA-bound particles was drop-cast onto carbon-coated copper TEM grids (formvar support, 300-mesh, Ted Pella). Bright-field images were captured digitally with Digital Micrograph using a Gatan 780 DualVison camera.

**High resolution Transmission Electron Microscopy**
High resolution electron microscopy was performed at the Shared Materials Instrumentation Facility, Duke University using a Hitachi HF-2000 FEG TEM operation at 200kV accelerating voltage. To prepare samples for analysis, an aqueous solution of RNA-bound particles was drop-cast onto carbon-coated copper TEM grids (formvar support, 300-mesh, Ted Pella).
**Inductively Coupled Plasma Mass Spectrometry (ICP-MS)**
ICP-MS analysis was performed by West Coast Analytical Service facility. For best results incubation reactions were scaled up at least 5x and the solid material was separated on 100 kDa molecular weight cut off filters, washed four times with dH2O and resuspended, followed by magnetic separation.

**Electron Energy Loss Spectroscopy (EELS)**
EELS was performed at Materials Research Laboratory at the Pennsylvania State University using field emission JEOL 2010F operating at 200 keV accelerating voltage. To prepare samples for analysis, an aqueous solution of RNA-bound particles was drop-cast onto carbon-coated copper TEM grids (formvar support, 300-mesh, Ted Pella).

**Preparation of truncated A5T1**
Truncate A5T1 was generated via PCR (see “Amplification of DNA Templates”) using a 22 bp primer 3’AATATGTAGGAAGAAGCTAAGGT5’, ordered from Midland Certified, Inc., complementary to the template 24 bases upstream in relation to the regular primer binding site. Resulting truncated PCR product was purified on 6% native polyacrylamide gel electrophoresis (PAGE) gel and then used as templates for in vitro transcription.
2.4 References


CHAPTER 3: ENGINEERING OF E. COLI TO PRODUCE MAGNETIC IRON OXIDE NANOPARTICLES

In Chapters 1 and 2 it has been described how imidazolyl-modified and native RNA sequences that mediate the formation of magnetic iron oxide nanoparticles were isolated. Based on this discovery it could be argued that these sequences could also function in vivo. Generating bacterial factories of magnetic iron oxide would allow for the scale up of the material and therefore make the material characterization by conventional methods possible. It will be shown how one of the native RNA sequences described in Chapter 2 was genetically encoded into E. Coli to synthesize the iron oxide nanoparticles in vivo.

3.1 Results and discussion

The native RNA isolate chosen for this project was A5 and the experimental strategy was to genetically encode A5 sequence into E.Coli via pET16b plasmid. Figure 3.1 shows the cloning region of pET16b vector. The plasmid was designed to contain the T7 RNA polymerase promoter sequence directly upstream from the A5 sequence and followed by the T7 terminator sequence. Since the DNA template of isolate A5 already contained a T7 promoter region, that part of the plasmid had to be removed using Bgl II and Xho I restriction sites. Because the T7 terminator sequence would add about 50 bases to the desired A5 transcript, the hepatitis delta ribozyme sequence (HδR) was inserted between A5 and the T7 terminator. HδR, after its transcription, folds on itself and splices the transcript one base after the A5 sequence.1,2 This self cleaving property of the ribozyme assisted in obtaining A5 transcript of desired length.
In order to test whether transcription of the recombinant plasmid would yield the desired A5 transcript the pET16bH\(\delta\)RA5 plasmid was subjected to *in vitro* transcription. A successful reaction would confirm the presence of the A5 construct and also demonstrate hepatitis \(\delta\) ribozyme splicing activity. A 6% denaturing PAGE was performed on the obtained product. There are three bands on the gel (Figure 3.2) corresponding to: (1) the full length transcription product containing A5 H\(\delta\)R and T7 terminator sequence (~298 bases), (2) the longer splicing product containing H\(\delta\)R_T7term (~211 bases), (3) product of interest, identical length of A5 isolate (87 bases) as compared to the control lane A which contains only A5 RNA.

Next transcription *in vivo* was tested. BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE bacterial strains were transformed with the recombinant plasmid pET16bH\(\delta\)RA5. Liquid cultures were induced with IPTG to initiate transcription and the bacteria were allowed to grow for 4 hours. The obtained bacterial mass was collected by centrifugation. The cells were lysed using 0.5 mg/mL of lysozyme. The total RNA was isolated employing a hot phenol/chloroform extraction, followed by ethanol precipitation. To remove any DNA contaminants DNase treatment was employed. The total RNA isolated from BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE bacterial strands was analyzed on 6% denaturing PAGE (Figure 3.3). Each sample contained the correct length transcript of interest, as compared to the A5 RNA generated *in vitro*. In addition, each sample contained high molecular weight material corresponding to cellular RNA. The samples were then subjected to RT-PCR reaction and the correct size PCR product was obtained. The PCR product was sequenced to confirm that the sequence was of A5 isolate.
Before attempting to synthesize the iron oxide nanoparticles \textit{in vivo} it had to be tested whether the bacteria can survive and properly function in an iron rich environment. Two independent viability assays were conducted to determine cell viability. In the first experiment the bacterial strain BL21(DE3) was transformed with the plasmid containing green fluorescent protein (GFP) gene, pET16bSGFP. In properly functioning cells this gene is expressed and the cells produce the fluorescent protein. Those bacteria were then inoculated into liquid medium containing three different iron concentrations: 1 mM, 0.1 mM and 0.05 mM. A no iron control was also performed. After overnight incubation at 37°C the bacterial cells were collected and imaged under blue light to detect the green fluorescence. Each bacterial sample produced the green fluorescent protein. As expected the most intense fluorescence was obtained for culture grown without the iron and it slightly decreased as the iron concentration increased. This result indicated that increasing iron concentrations slightly decreased the production of GFP by bacteria however the effect was minor indicating that the bacteria survived and functioned properly. The other iron toxicity assay that was conducted involved growing bacterial colonies on agar containing 1 mM FeCl₂ and 0.1 mM sodium citrate. All three bacterial strains were tested BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE. They were transformed with pET16bHδRA5 engineered plasmid. As a control the bacteria was also grown on regular LB plates. After overnight incubation in 37°C there was no difference in bacterial growth on the plates with and without iron confirming the results from the previous experiment that 1 mM iron concentration did not inhibit the bacterial growth. It has to be noted that in the GFP experiment the bacteria survived in 1 mM iron concentrations despite the lack of iron sequestering ability. In contrast during the second test the presence of the engineered plasmid pET16bHδRA5 in the bacteria decreased the iron toxicity in the cell by utilizing the iron for particle synthesis, as will be shown below.

Since both \textit{in vitro} and \textit{in vivo} transcriptions were successful the next step was to transform bacteria with the recombinant plasmid and attempt \textit{in vivo} iron oxide nanoparticle synthesis. The engineered plasmid, pET16bHδRA5 was transformed into three different strains of BL21 bacteria: BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE. Liquid cultures were induced with IPTG (0.5 mM) to initiate transcription, followed by addition of FeCl₂ to $[I]_f = 1$ mM, and sodium citrate to $[C]_f = 0.1$ mM and allowed to grow overnight at 37°C. Citrate was previously found to increase the iron uptake into the bacterial cells.\textsuperscript{3} Next
the cells were collected and lysed using 0.5 mg/mL lysozyme. The particles were isolated by means of magnetic partitioning and analyzed via TEM. All three bacterial strands yielded an abundance of spherical particles. Figure 3.4A illustrates the material synthesized by the BL21(DE3) bacterial strain, the particles were less than 3 nm in size. Cryo-electron tomography was also used to analyze the material synthesized with BL21(DE3)pLysS bacteria containing engineered plasmid pET16bHδRA5. As shown in Figure 3.4B abundance of nanoparticles about 3 nm in size was observed. In an attempt to characterize the material isolated from bacteria, nanoparticles were analyzed by energy dispersive x-ray spectroscopy (EDS) and an iron peak was detected as can be seen in figure 3.5. Two control experiments were performed to show that the electron-dense material observed by TEM was the product of an interaction between FeCl₂ and the A5 RNA sequence. First, bacteria containing the pET16bHδRA5 plasmid were cultured in the absence of FeCl₂. Second, bacteria transformed with 3 different plasmids (pET16HδR, pet16b containing TFII transcription factor and pUC18) all lacking the A5 insert were incubated in FeCl₂. In all controls, we were unable to locate the nanoparticles. This suggested that the electron-dense material observed in Figure 3.4 originated from an interaction between A5 and Fe²⁺.

Figure 3.4 A - TEM image of the material synthesized by BL21(DE3) transformed with the engineered plasmid pET16bHδRA5; B – cryo-electron tomography of material synthesized by BL21(DE3)pLysS transformed with pET16bHδRA5.
To further prove that the A5 sequence was involved in nanoparticle formation, the particles were subjected to RT-PCR. BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE cells were induced with IPTG and grown overnight in the presence of 1 mM FeCl₂ and 0.1 mM sodium citrate. The collected pellets were lysed with lysozyme and magnetic partitioning was performed to isolate the iron oxide particles. Next the samples were treated with DNase to remove any possible DNA contamination. After the DNase digest the samples were purified by magnet partitioning to remove the enzyme. The samples were then subjected to the RT-PCR reaction. The correct length of PCR product was obtained from each sample. The PCR product was sequenced and it was confirmed that the sequence was of the A5 isolate. Control experiments in which pET16bHδRA5 plasmid was replaced with pET16b lacking A5 insert, or pUC18, and treated analogously failed to yield any RT/PCR product. When bacteria were incubated in the absence of FeCl₂ no RT-PCR product was detected.

Figure 3.5 EDS spectrum of nanoparticles isolated from BL21(DE3) bacteria.
3.2 Summary

Isolate A5, recovered from a native RNA in vitro selection (Chapter 2), was genetically encoded into E. Coli via the pET16b plasmid. The resulting bacteria produced A5 RNA and when grown in presence of 1 mM of FeCl₂ and 0.1 mM of sodium citrate they generated spherical, iron containing nanoparticles, about 3 nm in size. It was shown that both iron and engineered plasmid pET16BH₅RA5 were required for the nanoparticle synthesis. The RT-PCR off of the nanoparticles yielded dsDNA product of the A5 isolate sequence, which proved that the A5 RNA was involved in the nanoparticle formation.

The ability of E. Coli to synthesize the iron oxide nanoparticles is of great advantage, since it will enable the material synthesis scale up. Already enough material has been generated to see it move in a vial in response to magnetic field. Currently work is being done to scale up the material synthesis for characterization by powder X-ray spectroscopy and Superconductive Quantum Interference Device (SQUID).
3.3 Experimental section

Reagents
DH5α containing pET16b, FHV containing hepatitis δ rybozyme, pET16b-SGFP palsmid

Vector preparation
The T7 promoter region was removed from pET16b vector utilizing Bgl II and Xho I restriction sites. In order to test the digestion efficiency test reactions were performed with each restriction enzyme individually. In Bgl II reaction 0.2 µg of the plasmid was combined with 1x NEB 3 buffer (pH 7.9, 50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 1 mM Dithiothreitol) and 0.5 U/µL of Bgl II enzyme New England Biolabs). Analogously in Xho I reaction 0.2 µg of the plasmid was combined with 1x NEB 2 buffer (10 mM Tris-HCl 50 mM NaCl, 10 mM MgCl₂, 1 mM Dithiothreitol, pH 7.9) and 1 U/µL of Xho I enzyme (New England Biolabs). Samples were incubated at 37°C for 1h. To confirm the digest samples were combined with 2 µL of 6x agarose loading dye (Fisher) and loaded on 7% agarose gel and run at 8 W for 3h. The linearized samples had slower mobility on the gel compared to a non digested control, Fig 3.5. Next a sequential bulk digest was performed. Five µg of the plasmid were digested with 0.6 U/µL Bgl II in presence of 1x NEB 3 buffer at 37°C for 1h. The digested plasmid was purified using QIAquick PCR Purification Kit (Qiagen). Purified linearized plasmid was then digested with 0.6 U/µL of Xho I restriction enzyme, in the presence of 1x NEB 2 buffer, at 37°C for 1h. As a result a 173 bp fragment was removed from the pET16b plasmid. To separate the digested plasmid from the excised fragment sample was loaded on a 7% agarose gel. The bands of interest were excised and electroeluted at a constant 2 W per sample for 3 hours followed by purification using QIAquick PCR Purification Kit (Qiagen) and quantitation via UV-Vis spectroscopy.
**Hepatitis δ rybozyme DNA template preparation**

FHVI plasmid containing the hepatitis δ rybozyme sequence was received, courtesy of Dr. Tim L. Sit from Department of Plant Pathology. To retrieve the rybozyme sequence by PCR following primers were ordered from Midland Certified, Inc.:

5RP 5’AGGCAGATCTCCCGGGTGCGATGGCATCTCTCCCA3’,
3RP 5’CTGGCTCGAGCGAGCTCTCCCTTAGCCATC3’.

Each primer contained 20 bases complementary to the plasmid and additional bases that allowed the introduction of the Bgl II restriction site at the 5’ end and Xho I site at 3’ end. Additionally at the 5’ end between the BglII site and the rybozyme sequence the Sma I restriction site was introduced, which was unique to the insert and which later served as a quick test for insert presence in the plasmid after insert ligation. To retrieve the rybozyme sequence from the FHVI plasmid 0.6 µg of the plasmid was combined with 1× Taq DNA Polymerase buffer (New England Biolabs, 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl, pH 8.8, 2 mM MgSO4, 0.1% Triton X-100), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.6 µM each 5RP and 3RP primers and 0.025 U/µL of Pfu Turbo DNA Polymerase (Stratagene). Twenty five cycles of PCR were conducted. To separate the PCR product from the FHVI plasmid the samples were loaded onto a 6% native PAGE and the band of interest was excised and electroeluted followed by purification using QIAquick PCR Purification Kit (Qiagen) and quantitation via UV-Vis spectroscopy. As a result the hepatitis δ rybozyme obtained:

5’AGGCAGATCTCCCGGGTGCGATGGCATCTCCACCTCCTCGGCACCTGGCATCCGAAAGGAGCGTCGACTCCACTCGGATGGCTAAGGGAGAGCTCGCTCGAACCCAG3’ (underlined are the portions of the sequence added for cloning purposes).

Next the rybozyme was sequentially digested with Bbl II and Xho I (see “Vector preparation”) to create sticky ends compatible with those on the pET16b vector. After each digest reaction the rybozyme was purified using QIAquick PCR Purification Kit (Qiagen) and quantitated via UV-Vis spectroscopy.
**Hepatitis δ rybozyme ligation into pET16b**

The hepatitis δ rybozyme (5 µg) was ligated into a previously digested pET16b (160 ng) vector using 0.15 U/µL of T4 ligase (Promega), in the presence of 1x ligase buffer (30mM Tris-HCl, pH 7.8, 10mM MgCl₂, 10mM DTT and 1mM ATP), at 22°C for 1h followed by enzyme deactivation at 65°C for 10 min. To grow more of the recombinant plasmid, pET16bHδR, it was used to transform and plate DH5α cells (for transformation protocol see “Total RNA harvest/Transformation). After an overnight incubation at 37°C 16 colonies were inoculated into 2 mL of liquid LB and incubated over night again at 37°C shaking at 200 rpm. Next the plasmid was isolated from each bacterial colony using Strata Prep Plasmid Miniprep Kit. To test for the presence of the HδR the plasmids were digested with the SmaI restriction enzyme (New England Biolabs). Hundred and fifty (150) ng of the plasmid was combined with 1x NEB 4 buffer (20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM Dithiothreitol, pH 7.9) and 0.4 U/µL of Sma I and incubated at 25°C for 1h. The samples were combined with 2 µL of 6x agarose loading dye (Fisher) then loaded onto a 7% agarose gel to detect the Sma I digest. Samples number 8, 11, 15 and 16 were digested and were sent for sequencing (MWG-Biotech AG) to confirm the presence of the HδR in the plasmid. The sequencing results confirmed the presence of the rybozyme for both samples 8 and 16. These samples were taken forward.

**Isolate A5 DNA template preparation**

For cloning purposes the Bgl II and the Stu I restriction sites were incorporated into the sequence at the 5’ end of the sequence by means of PCR using a modified 5’ primer 5P8BglStu: 5’AGGCAGATCTAGGCCTTAATACGACTCACTATAGGG 3’, purchased from Midland Certified, Inc. Twenty (20) cycles of PCR were performed using 0.35 µg of the A5 DNA template, 1× Taq DNA Polymerase buffer (New England Biolabs, 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl, pH 8.8, 2 mM MgSO4, 0.1% Triton X-100), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 0.6 µM each 5P8BglStu and 3RP primers and 0.025 U/µL of Pfu Turbo DNA Polymerase (Stratagene). After the reaction the A5 construct was gel purified and digested with Bgl II (see “Vector preparation”) to create the sticky end at the 5’ of the fragment. The digested sample was purified with QIAquick PCR Purification
Kit (Qiagen) and quantitated via UV-Vis spectroscopy. The 3’ end of the A5 sequence remained blunt to be compatible with the blunt Sma I site created on the vector.

**A5 construct ligation into pET16bHδR**

To prepare the pET16bHδR vector for ligation a 6 µg aliquot was first digested with Bgl II enzyme (see “Vector preparation”) and purified with QIAquick PCR Purification Kit (Qiagen). Next the vector was digested by 1.2 U/µL Sma I (New England Biolabs) in presence of 1x NEB 4 buffer (20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM Dithiothreitol, pH 7.9) at 25°C for 1h. The digested vector was purified with QIAquick PCR Purification Kit (Qiagen) and quantitated via UV-Vis spectroscopy. Sixty eight (68) ng of the A5 construct was ligated, in separate experiments, to the pET16bHδR vectors 8 and 16 (400 ng each) using 0.15 U/µL T4 ligase (Promega) and 1x ligase buffer (Promega). To grow more of the recombinant plasmid pET16bHδRA5. DH5 α cells were transformed with the plasmid and plated onto agar plates (for transformation protocol see “Total RNA harvest/Transformation). After an overnight incubation at 37°C 20 colonies were picked (1a-10a from plasmid pET16bHδR #8 and 11a-20a from plasmid pET16bHδR #16), inoculated into 2 mL of liquid LB and incubated over night at 37°C, shaking at 200 rpm. The bacteria were then harvested and the plasmids were isolated using the Strata Prep Plasmid Miniprep Kit. To check for the A5 insert presence the plasmids were digested with Stu I. Only sample 10a passed the digest test. To confirm the presence of the A5 insert plasmid 10a was sent for sequencing.

**In vitro transcription**

First the 4.2 µg of pET16bHδRA5 plasmid was linearized downstream from A5HδR_ T7term construct with 1.2 U/µL of the Hind III restriction enzyme (New England Biolabs) in presence of 1x NEB 2 buffer at 37°C for 9h. The linearized plasmid was purified using QIAquick PCR Purification Kit (Qiagen). Next transcription was performed using a Megascript Kit (Ambion) at 37°C for 9 hours using 0.5 µg of linearized pET16bHδRA5 plasmid, ATP, CTP, GTP, UTP (8 mM each), 1x reaction buffer and enzyme mix (Megascript®, Ambion).
Total RNA harvest

Transformation: 50 µL aliquots of each bacterial strands: BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE) were thawed on ice and transferred to chilled 10-mL falcon tube. Ten (10) ng of pET16bHδRA5 plasmid DNA was added to the cells and gently mixed. The vials were incubated on ice for 30 min. Next the cells were heat pulsed in a 42 °C water bath for 30 seconds. Following the heat pulse, the cells were placed on ice; then 0.25 mL of preheated (42 °C) SOC medium was added to the cells and incubated at 37 °C for 1 hour with shaking at 225 rpm. A sterile spreader was used to plate the transformed cells onto already prepared LB Amp agar plates containing 50 µg/mL ampicilin. Additionally 34 µg/mL of chloroamphenicol were added to the plates for pLysS and pLysE containing bacteria. The plates were incubated at 37 °C for 15-18 hours. The obtained colonies were picked and inoculated into 2 mL liquid LB Amp Agar (Fermentas) and incubated overnight at 37 °C with shaking at 225 rpm. Those saturated liquid cultures were stored as stock cultures.

In vivo RNA production and isolation A 300 µL aliquot of saturated cultures was added to 2 mL of fresh LB and grown 37 ºC with shaking at 225 rpm until the OD_{600} of the cultures reached 0.5. To initiate transcription 1 mM IPTG was added and the cultures were allowed to grow for another 4 hours. To harvest cells 2 mL of liquid cultures was combined with 0.25 mL of ice-cold EtOH/Phenol stop solution (5% water-saturated phenol, pH<7.0), to stop RNA degradation. The bacterial matter was collected by centrifugation. The pellets were resuspended in 0.2 mL of TE buffer (composition, pH 8.0). The cells were lysed using 0.5 mg/mL of lysozyme at 64°C for 2 min. After incubation NaOAc, pH 5.2 was added to a final concentration of 100 mM. Next to perform a hot phenol extraction the samples were added to an equal volume of water saturated phenol (pH<7), inverted 10 times (every 40 s) and cubated in 64°C for 6 min. The tubes were then placed on ice to chill and centrifuged at max speed for 10 min at 4°C. The aqueous layer was transferred to fresh tube containing an equal volume of chloroform, inverted 10 times and centrifuged at 14000 rpm for 5 min at 4°C. The aqueous layer of each sample was equally divided into two tubes, then one tenth volume of 3 M NaOAc pH 5.2 1 mM EDTA and 2.5 volumes of cold 100% EtOH were added, incubated at -80°C for 20 min. After incubation the samples were centrifuged at 14000 rpm for 25 min at 4°C. The ethanol was carefully removed and a small while pellet remained. The pellet was washed with 1 mL of 80% cold ethanol, centrifuged at 14000 rpm for 5 min., the ethanol was
carefully removed and pellet was air dried. To remove any DNA contamination the samples were treated with 0.04 U/µL DNase (New England Biolabs) in presence of 1x DNase I buffer (10 mM MgCl₂, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT) and 0.08 U/µL RNase inhibitor (Promega), at 37°C for 30 min. Next RNA was purified by phenol extraction, followed by phenol/chloroform (50:50) and chloroform extractions followed by ethanol precipitation. The pellet was resuspended in 50 µL of RNase-free H₂O. Obtained total RNA was loaded onto a 6% denaturiung PAGE.

**Iron oxide synthesis in vivo**

Ten (10) µL aliquots of liquid cultures: BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE; containing the pET16bHδRA5 recombinant vector; were inoculated into 3 mL of fresh LB medium and grown till saturation (OD₆₀₀=>2) overnight at 37°C. Next 3 mL of freshly prepared medium were inoculated with 60 µL of the saturated culture and the cultures were grown until they reached the mid-log phase (OD₆₀₀= ~0.5, 4 to 5 hours). To induce transcription IPTG was added to final concentration of 0.5 mM, followed by addition of FeCl₂ to [f] = 1 mM, and sodium citrate to [f] = 0.1 mM. The bacteria were allowed to grow at 37°C overnight. The cells were then collected by centrifugation and the LB was removed. The pellets were resuspended in 200 µL of TE buffer (10 mM TrisHCl, 1 mM EDTA, pH 8). To lyse the cells a lysozyme was added to a final concentration of 10 mg/mL and the sample was incubated at 30°C for 30 min. Particles were isolated by means of magnetic partitioning and analyzed by TEM.

**RT-PCR off of the particles**

Particles isolated from bacterial preparations (see “Iron oxide synthesis in vivo”) were treated with DNase to remove any contaminating DNA; 0.04 U/µLDNase (New England Biolabs) was used in the presence of 1x DNase I buffer (10 mM MgCl₂, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT) and 0.08 U/µL RNase inhibitor (Promega), at 37°C for 30 min. The particles were then purified by magnet partitioning. To perform RT-PCR the resuspended nanoparticles were combined with 1x Syber Green RT-PCR mix (Bio-Rad) and 0.3 µM 3P8 and 5P8 primers, incubated at 50°C for 10 min followed by 95°C for 5 min., followed by 22 cycles of of 95 °C, 30 seconds, 60 °C, 30 seconds, 72 °C, 45 seconds. The
RT-PCR products were purified by washing 4x on size-exclusion membranes (Microcon 30, 30-kD cutoff) with DEPC H₂O and resuspended in 70 µL of DEPC H₂O and quantitated by UV-Vis spectroscopy. To confirm that the RT-PCR product was of the correct sequence it was cloned into pPCR-Script™ Amp SK(+) cloning vector and sent off for sequencing to University of Michigan Core Sequencing Facility.

Cloning procedure

RT-PCR product preparation: The samples were amplified using standard PCR conditions (see “Amplification of DNA Templates”), incorporating a custom 5′-primer 5′ Cloning Primer (5CP, 5'-GGGAGACAAGAATAAACGCTCAA-3'), which was synthesized by Midland Certified, Inc. The amplification product was purified on a 6% PAGE; the ds5CP-DNA product was excised (how did I know which band to excise) from the gel and electroeluted at a constant 10 W for 4 hours. Following electroelution, the ds5CP-DNA was purified using a QIAGen PCR Purification Kit (Qiagen). The resulting ds5CP-DNA was quantitated by UV-Vis spectroscopy.

Polishing: Following the procedure provided with the PCR-Script™ Amp Cloning Kit (Stratagene) the ds5CP-DNA sample was polished using cloned 0.04 U/µL Pfu DNA Polymerase, 1× polishing buffer and 0.19 mM each of dATP, dCTP, dGTP, and dTTP. The polishing reaction was mixed gently and then incubated at 72 °C for 30 minutes. Pfu DNA polymerase generated high fidelity, blunt-ended PCR products.

Ligating the Insert to Vector: Blunt-ended ligation was performed by incubating 0.3 µM polished 5CP-DNA with the predigested 1 ng/µL pPCR-Script™ Amp SK(+) cloning vector (ca. 30 kDa) (Stratagene), 1× reaction buffer, 0.5 mM rATP, 0.5 U/µL Srf I restriction enzyme, 0.4 U/µL T4 DNA Ligase, and water to a final volume of 10 µL. The ligation reaction was mixed gently and then incubated at 25 °C for 2 hours. Following the incubation, the ligase was inactivated by heating at 65 °C for 10 minutes.

Transformations: XL10-Gold Kan® Ultracompetent cells (Stratagene) were thawed on ice and gently mixed by tapping. A 40 µL aliquot of cells was added to a chilled 10-mL falcon tube and a 1.6 µL aliquot of XL10-Gold β-mercaptoethanol mix (Stratagene) was added to the cells. The cells were swirled gently and incubated on ice for 10 minutes, with swirling every 2 minutes. A 2 µL aliquot of the ligation product was then added to the cells. The cells were incubated on ice for an additional 30 minutes. Following this incubation, the cells were
heat pulsed in a 42 °C water bath for 30 seconds (the temperature and length of heat pulse was critical for obtaining the highest efficiencies). Following the heat pulse, the cells were placed on ice for 2 minutes; then 0.45 mL of preheated (42 °C) NZY+ broth was added to the cells and incubated at 37 °C for 1 hour with shaking at 250 rpm. A sterile spreader was used to plate the transformed cells onto already prepared LB Amp agar (Fermentas) plates containing 50 µg/mL of ampicilin and for blue white color screening 100 µg/mL of IPTG and 100 µg/mL of X-Gal. The plates were incubated at 37 °C for 15-18 hours. To insure that obtained white colonies do not contain multiple inserts, individual white colonies were picked with sterile toothpicks and streaked onto prepared LB Amp agar plates containing IPTG and X-Gal. These plates were then incubated for 15-18 hours at 37 °C. Following this incubation, the plates were placed at 4 °C to enhance the white color of the colonies.

Viability tests

Green fluorescent protein expression

BL21(DE3) bacterial strand was transformed with the pET16bSGFP plasmid that contained gene for green fluorescent protein (see “Total RNA harvest/Transformation” for the protocol). After transformation the cells were plated onto already prepared LB Amp agar plates containing ampicilin (50 µg/mL). The plates were incubated at 37 °C for 15-18 hours. The obtained colonies were picked and inoculated into three separate 3 mL liquid LB Amp Agar (Fermentas) containing 1 mM, 0.1 mM and 0.05 mM FeCl₂ respectively, and incubated overnight at 37 °C with shaking at 225 rpm. To collect bacterial mass the samples were centrifuged at 10000 rpm for 10 min and resuspended in 100 µL of 1x TBS buffer (10 mM TRIS, 15 mM NaCl, pH 7.4). The resuspended cells were imaged in a blue light to test for the green fluorescence.

Bacterial growth on Fe rich medium

BL21(DE3), BL21(DE3)pLysS and BL21(DE3)pLysE bacterial strands were transformed with the engineered plasmid pET16bHδRA5 and plated onto already prepared LB Amp agar plates containing ampicilin (50 µg/mL). Additionally 34 µg/mL of chloroamphenicol were added to the plates for pLysS and pLysE containing bacteria. The plates were incubated at 37 °C for 15-18 hours. The obtained colonies were streaked onto LB Amp agar plate containing 1 mM FeCl₂ and 0.1 mM sodium citrate. For side by side comparison the bacteria was plated
onto regular LB Amp agar plate. After overnight incubation at 37 °C the colonies growth was compared.

**Transmission Electron Microscopy**

Bright-field images were obtained using TEM at the University of North Carolina School of Dentistry using a Philips CM12 transmission electron microscope operating at 100 kV accelerating voltage. To prepare samples for analysis, an aqueous solution of RNA-bound particles was drop-cast onto carbon-coated copper TEM grids (formvar support, 300 mesh, Ted Pella). Bright-field images were captured digitally with Digital Micrograph using a Gatan 780 DualVison camera.

**Energy Dispersive X-ray Spectrometry**

EDS was performed at the Shared Materials Instrumentation Facility, Duke University using a Hitachi HF-2000 FEG TEM equipped with an Oxford Instruments Inca Energy 100 energy dispersive x-ray spectrometer. To prepare samples for EDS analysis, an aqueous solution of RNA-bound particles was drop-cast onto carbon-coated copper TEM grids (formvar support, 300 mesh, Ted Pella).
3.4 References


CONCLUSIONS

Metal oxides are an important class of materials used commercially in applications such as catalysis, solar energy conversion, magnetic information storage, and chemical sensing. In the biosphere metal oxides have been found to act as building blocks, provide protection against predation, also many oxide materials found in nature have sophisticated physical properties such as magnetism and light focusing.

Here it has been shown that RNA sequences that mediate the formation of magnetic iron oxide nanoparticles were isolated from large, native or imidazolyl modified, random RNA sequence libraries. The active sequences were isolated using new magnetic field partitioning protocol. This is the first example of in vitro selection based on a property of the material that is being synthesized. The success of this study presents new opportunities in inorganic materials chemistry. Using in vitro selection to explore the ability of RNA for the assembly of inorganic materials has revealed possibilities for the creation of new materials possessing the desired property.

Furthermore, it has been shown that E.Coli could be genetically modified to become iron oxide factories enabling for the material scale up. Preliminary results suggest that genetically encoding the RNA sequence capable of mediating the formation of iron oxide into the bacterial plasmid decreases the toxic effect of the iron on the bacterial growth. This methodology has a potential for developing similar systems that could be utilized for heavy metal sequestration. Additionally the ability to synthesize inorganic particles inside of the bacterial cell presents a great potential for their use as a contrasting markers for biological electron microscopy.

Many questions still remain unanswered. What is the mechanism of the RNA mediated iron oxide synthesis? What is the secondary structure of the active RNA sequences? What is the number of RNA molecules present per nanoparticle? The composition of the synthesized material has been determined to be magnetite (Fe₃O₄), or maghemite (γ-Fe₂O₃), or a mixture of those. The other possibility is that for Fe(II)/Fe(III) ratio smaller than 0.5 a spinel oxide could form containing cation vacancies. Nevertheless the exact composition of the synthesized material needs to be determined. Finally, it is also imperative to characterize the magnetic properties of the material since it has been shown to
respond to the magnetic field. Studies are now taking place to gain deeper understanding of the discussed system.
Table A1 Experimental and theoretical d values comparison for selected area diffraction from experiment 1, (hkl)(theoretical d value)(% error).

<table>
<thead>
<tr>
<th>Experimental d values</th>
<th>Magnetite (Fe₃O₄)</th>
<th>Hematite (α-Fe₂O₃)</th>
<th>Maghemite (γ-Fe₂O₃)</th>
<th>α-FeOOH</th>
<th>β-FeOOH</th>
<th>NaCl</th>
<th>KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.116</td>
<td>(200) (4.198) (1.95%)</td>
<td>X</td>
<td>(200) (4.17) (1.2%)</td>
<td>(110) (4.18) (1.5%)</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>3.071</td>
<td>(220) (2.97) (3.4%)</td>
<td>X</td>
<td>(220) (2.95) (4%)</td>
<td>X</td>
<td>(100) (3.08)</td>
<td>X</td>
<td>(200) (3.13) (1.8%)</td>
</tr>
<tr>
<td>2.676</td>
<td>(310) (2.655) (0.79%)</td>
<td>X</td>
<td>(104) (2.71) (1.2%)</td>
<td>(130) (2.693) (0.6%)</td>
<td>X</td>
<td>(200) (2.815) (4.7%)</td>
<td>X</td>
</tr>
<tr>
<td>1.876</td>
<td>(420) (1.877) (0%)</td>
<td>X</td>
<td>(331) (1.91) (1.8%)</td>
<td>X</td>
<td>X</td>
<td>(300) (1.877) (0%)</td>
<td>X</td>
</tr>
<tr>
<td>1.611</td>
<td>(511) (1.616) (0.3%)</td>
<td>(211) (1.637)</td>
<td>(511) (1.605) (0.4%)</td>
<td>(151) (1.564) (3%)</td>
<td>(112) (1.625)</td>
<td>X</td>
<td>(400) (1.565) (3.5%)</td>
</tr>
<tr>
<td>1.527</td>
<td>(521) (1.533) (0.4%)</td>
<td>(300) (1.454)</td>
<td>(440) (1.475) (3.5%)</td>
<td>(002) (1.511) (1%)</td>
<td>(210) (1.528)</td>
<td>X</td>
<td>(400) (1.56) (2.1%)</td>
</tr>
<tr>
<td>1.202</td>
<td>(444) (1.266) (0.8%)</td>
<td>(311) (1.205)</td>
<td>X</td>
<td>(400) (1.152) (4.3%)</td>
<td>(420) (1.259) (4.5%)</td>
<td>(511) (1.205)</td>
<td></td>
</tr>
<tr>
<td>1.085</td>
<td>(553) (1.083) (0.2%)</td>
<td>(411) (1.091)</td>
<td>X</td>
<td>(411) (1.07) (1%)</td>
<td>X</td>
<td>(511) (1.083) (0.2%)</td>
<td>(440) (1.107) (2%)</td>
</tr>
</tbody>
</table>
Table A2 Experimental and theoretical d values comparison for selected area diffraction from experiment 2, (hkl)(theoretical d value)(% error).

<table>
<thead>
<tr>
<th>Experimental d values</th>
<th>Magnetite (Fe₃O₄)</th>
<th>Hematite (α-Fe₂O₃)</th>
<th>Maghemite (γ-Fe₂O₃)</th>
<th>α-FeOOH</th>
<th>β-FeOOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental d values</td>
<td>Theoretical d values</td>
<td>% error</td>
<td>Theoretical d values</td>
<td>% error</td>
<td>Theoretical d values</td>
</tr>
<tr>
<td>2.7060</td>
<td>(310) (2.655) (1.9%)</td>
<td>X</td>
<td>(104) (2.71) (0.1%)</td>
<td>(130) (2.693) (0.5%)</td>
<td>X</td>
</tr>
<tr>
<td>2.0413</td>
<td>(400) (2.08) (1.9%)</td>
<td>(116) (2.087) (2.1%)</td>
<td>(140) (2.023) (0.9%)</td>
<td>(220) (2.9) (2.4%)</td>
<td>X</td>
</tr>
<tr>
<td>1.7623</td>
<td>(422) (1.714) (2.8%)</td>
<td>X</td>
<td>(422) (1.703) (3.5%)</td>
<td>(221) (1.71) (2.9%)</td>
<td>(151) (1.735) (1.4%)</td>
</tr>
<tr>
<td>1.265</td>
<td>(533) (1.2688) (0.3%)</td>
<td>(221) (1.254) (0.9%)</td>
<td>(533) (1.272) (0.6%)</td>
<td>X</td>
<td>(003) (1.29) (1.9%)</td>
</tr>
<tr>
<td>1.0675</td>
<td>(553) (1.083) (1.7%)</td>
<td>(012) (1.036) (2.8%)</td>
<td>(553) (1.086) (1.7%)</td>
<td>(411) (1.07) (0.3%)</td>
<td>(300) (1.027) (3.9%)</td>
</tr>
</tbody>
</table>

Table A3 Experimental and theoretical d values comparison for selected area diffraction from experiment 3, (hkl)(theoretical d value)(% error).

<table>
<thead>
<tr>
<th>Experimental d values</th>
<th>Magnetite (Fe₃O₄)</th>
<th>Hematite (α-Fe₂O₃)</th>
<th>Maghemite (γ-Fe₂O₃)</th>
<th>α-FeOOH</th>
<th>β-FeOOH</th>
<th>NaCl</th>
<th>KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental d values</td>
<td>Theoretical d values</td>
<td>% error</td>
<td>Theoretical d values</td>
<td>% error</td>
<td>Theoretical d values</td>
<td>% error</td>
<td></td>
</tr>
<tr>
<td>4.502 ± 0.013</td>
<td>X</td>
<td>(003) (4.59) (0.6%)</td>
<td>X</td>
<td>(100) (4.608) (2%)</td>
<td>X</td>
<td>X</td>
<td>(110) (4.4265) (1.7%)</td>
</tr>
<tr>
<td>2.613 ± 0.013</td>
<td>(310) (2.655) (1.6%)</td>
<td>(110) (2.519) (3.7%)</td>
<td>(130) (2.638) (0.9%)</td>
<td>(130) (2.69) (2.9%)</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>2.076 ± 0.028</td>
<td>(400) (2.08) (0.2%)</td>
<td>(116) (2.087) (0.5%)</td>
<td>(400) (2.86) (1.2%)</td>
<td>(220) (2.091) (0.7%)</td>
<td>X</td>
<td>X</td>
<td>(300) (2.087) (0.5%)</td>
</tr>
<tr>
<td>1.695 ± 0.013</td>
<td>(422) (1.71) (0.9%)</td>
<td>(210) (1.649) (2.8%)</td>
<td>(422) (1.703) (1.8%)</td>
<td>(221) (1.719) (1.4%)</td>
<td>X</td>
<td>(300) (1.877)</td>
<td>X</td>
</tr>
<tr>
<td>1.299 ± 0.013</td>
<td>(533) (1.2688) (2.4%)</td>
<td>(221) (1.254) (3.6%)</td>
<td>(116) (1.353) (4%)</td>
<td>(311) (1.357) (4.3%)</td>
<td>(003) (1.290) (0.7%)</td>
<td>(311) (1.698) (4.278)</td>
<td></td>
</tr>
<tr>
<td>1.217 ± 0.014</td>
<td>(444) (1.212) (0.4%)</td>
<td>(310) (1.210) (0.6%)</td>
<td>(444) (1.204) (1%)</td>
<td>X</td>
<td>X</td>
<td>(400) (1.408) (151) (1.205)</td>
<td></td>
</tr>
<tr>
<td>1.159 ± 0.012</td>
<td>X</td>
<td>(311) (1.205) (3.8%)</td>
<td>(444) (1.204) (3.7%)</td>
<td>(400) (1.152) (0.6%)</td>
<td>X</td>
<td>(420) (1.259)</td>
<td>X</td>
</tr>
<tr>
<td>1.057 ± 0.019</td>
<td>(800) (1.049) (0.8%)</td>
<td>(411) (1.091) (3.1%)</td>
<td>X</td>
<td>(322) (1.053) (0.4%)</td>
<td>X</td>
<td>(440) (0.995)</td>
<td>X</td>
</tr>
<tr>
<td>1.009 ± 0.013</td>
<td>(733) (1.026) (1.7%)</td>
<td>(104) (1.002) (0.7%)</td>
<td>X</td>
<td>(003) (1.007) (0.2%)</td>
<td>(301) (0.992) (1.7%)</td>
<td>X</td>
<td>(006) (1.006) (0.3%)</td>
</tr>
<tr>
<td>(hkl)</td>
<td>Magnetite (Fe3O4)</td>
<td>Hematite (α-Fe2O3)</td>
<td>Maghemite (γ-Fe2O3)</td>
<td>α-FeOOH</td>
<td>β-FeOOH</td>
<td>Fe(OH)₃</td>
<td>NaCl</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>100</td>
<td>8.396</td>
<td>4.363</td>
<td>8.342</td>
<td>4.608</td>
<td>3.08</td>
<td>7.544</td>
<td>5.63</td>
</tr>
<tr>
<td>110</td>
<td>5.937</td>
<td>2.519</td>
<td>5.899</td>
<td>4.182</td>
<td>2.991</td>
<td>5.340</td>
<td>3.981</td>
</tr>
<tr>
<td>001</td>
<td>8.396</td>
<td>13.772</td>
<td>8.342</td>
<td>3.022</td>
<td>3.87</td>
<td>7.558</td>
<td>5.63</td>
</tr>
<tr>
<td>061</td>
<td>1.380</td>
<td>0.726</td>
<td>1.371</td>
<td>1.454</td>
<td>1.834</td>
<td>1.243</td>
<td>0.926</td>
</tr>
<tr>
<td>012</td>
<td>3.755</td>
<td>1.036</td>
<td>2.731</td>
<td>1.494</td>
<td>1.912</td>
<td>3.380</td>
<td>2.518</td>
</tr>
<tr>
<td>102</td>
<td>3.755</td>
<td>3.685</td>
<td>3.731</td>
<td>1.436</td>
<td>1.638</td>
<td>3.379</td>
<td>2.518</td>
</tr>
<tr>
<td>044</td>
<td>2.036</td>
<td>1.002</td>
<td>2.023</td>
<td>0.746</td>
<td>0.923</td>
<td>1.833</td>
<td>1.365</td>
</tr>
<tr>
<td>113</td>
<td>2.531</td>
<td>3.385</td>
<td>2.515</td>
<td>0.979</td>
<td>1.184</td>
<td>2.278</td>
<td>1.698</td>
</tr>
<tr>
<td>116</td>
<td>1.362</td>
<td>2.087</td>
<td>1.353</td>
<td>0.5</td>
<td>0.631</td>
<td>1.226</td>
<td>0.913</td>
</tr>
<tr>
<td>130</td>
<td>2.655</td>
<td>1.210</td>
<td>2.638</td>
<td>2.693</td>
<td>2.477</td>
<td>2.390</td>
<td>1.780</td>
</tr>
<tr>
<td>140</td>
<td>2.036</td>
<td>0.952</td>
<td>2.023</td>
<td>2.190</td>
<td>2.194</td>
<td>1.833</td>
<td>1.365</td>
</tr>
<tr>
<td>151</td>
<td>1.616</td>
<td>0.782</td>
<td>1.605</td>
<td>1.564</td>
<td>1.735</td>
<td>1.455</td>
<td>1.083</td>
</tr>
<tr>
<td>200</td>
<td>4.198</td>
<td>2.182</td>
<td>4.171</td>
<td>2.304</td>
<td>1.54</td>
<td>3.772</td>
<td>2.815</td>
</tr>
<tr>
<td>211</td>
<td>3.428</td>
<td>1.637</td>
<td>3.406</td>
<td>1.802</td>
<td>1.422</td>
<td>3.082</td>
<td>2.298</td>
</tr>
<tr>
<td>112</td>
<td>3.428</td>
<td>2.366</td>
<td>3.406</td>
<td>1.421</td>
<td>1.625</td>
<td>3.085</td>
<td>2.298</td>
</tr>
<tr>
<td>210</td>
<td>3.755</td>
<td>1.649</td>
<td>3.731</td>
<td>2.245</td>
<td>1.528</td>
<td>3.375</td>
<td>2.518</td>
</tr>
<tr>
<td>220</td>
<td>2.97</td>
<td>2.505</td>
<td>2.95</td>
<td>2.091</td>
<td>1.495</td>
<td>2.670</td>
<td>1.991</td>
</tr>
<tr>
<td>221</td>
<td>2.799</td>
<td>1.254</td>
<td>2.781</td>
<td>1.719</td>
<td>1.395</td>
<td>2.518</td>
<td>1.877</td>
</tr>
<tr>
<td>300</td>
<td>2.799</td>
<td>1.454</td>
<td>2.781</td>
<td>1.536</td>
<td>1.027</td>
<td>2.515</td>
<td>1.877</td>
</tr>
<tr>
<td>311</td>
<td>2.531</td>
<td>1.205</td>
<td>2.49</td>
<td>1.357</td>
<td>0.989</td>
<td>2.275</td>
<td>1.698</td>
</tr>
<tr>
<td>310</td>
<td>2.655</td>
<td>1.210</td>
<td>2.638</td>
<td>1.518</td>
<td>1.023</td>
<td>2.386</td>
<td>1.78</td>
</tr>
<tr>
<td>301</td>
<td>2.655</td>
<td>1.446</td>
<td>2.638</td>
<td>1.369</td>
<td>0.992</td>
<td>2.386</td>
<td>1.78</td>
</tr>
<tr>
<td>003</td>
<td>2.799</td>
<td>4.590</td>
<td>2.781</td>
<td>1.007</td>
<td>1.290</td>
<td>2.519</td>
<td>1.877</td>
</tr>
<tr>
<td>322</td>
<td>2.036</td>
<td>0.991</td>
<td>2.023</td>
<td>1.053</td>
<td>0.898</td>
<td>1.831</td>
<td>1.365</td>
</tr>
<tr>
<td>324</td>
<td>1.559</td>
<td>0.961</td>
<td>1.549</td>
<td>0.672</td>
<td>0.700</td>
<td>1.403</td>
<td>1.045</td>
</tr>
<tr>
<td>331</td>
<td>1.926</td>
<td>0.838</td>
<td>1.914</td>
<td>1.357</td>
<td>0.965</td>
<td>1.733</td>
<td>1.292</td>
</tr>
<tr>
<td>333</td>
<td>1.616</td>
<td>0.826</td>
<td>1.605</td>
<td>0.816</td>
<td>0.789</td>
<td>1.454</td>
<td>1.083</td>
</tr>
<tr>
<td>422</td>
<td>1.714</td>
<td>0.819</td>
<td>1.703</td>
<td>0.901</td>
<td>0.711</td>
<td>1.541</td>
<td>1.149</td>
</tr>
<tr>
<td>420</td>
<td>1.877</td>
<td>0.825</td>
<td>1.865</td>
<td>1.122</td>
<td>0.764</td>
<td>1.688</td>
<td>1.259</td>
</tr>
<tr>
<td>411</td>
<td>1.979</td>
<td>1.091</td>
<td>1.966</td>
<td>1.07</td>
<td>0.754</td>
<td>1.779</td>
<td>1.327</td>
</tr>
<tr>
<td>400</td>
<td>2.08</td>
<td>1.091</td>
<td>2.086</td>
<td>1.152</td>
<td>0.77</td>
<td>1.886</td>
<td>1.408</td>
</tr>
<tr>
<td>440</td>
<td>1.484</td>
<td>0.630</td>
<td>1.475</td>
<td>1.045</td>
<td>0.748</td>
<td>1.335</td>
<td>0.995</td>
</tr>
<tr>
<td>444</td>
<td>1.212</td>
<td>0.619</td>
<td>1.204</td>
<td>0.612</td>
<td>0.592</td>
<td>1.090</td>
<td>0.813</td>
</tr>
<tr>
<td>511</td>
<td>1.616</td>
<td>0.782</td>
<td>1.605</td>
<td>0.878</td>
<td>0.608</td>
<td>1.452</td>
<td>1.083</td>
</tr>
<tr>
<td>521</td>
<td>1.533</td>
<td>1.523</td>
<td>0.868</td>
<td>1.378</td>
<td>1.028</td>
<td>1.143</td>
<td>1.083</td>
</tr>
<tr>
<td>533</td>
<td>1.2688</td>
<td>1.272</td>
<td>1.151</td>
<td>0.859</td>
<td>0.955</td>
<td></td>
<td></td>
</tr>
<tr>
<td>553</td>
<td>1.083</td>
<td>1.086</td>
<td>0.983</td>
<td>1.138</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>622</td>
<td>1.266</td>
<td>1.258</td>
<td>1.138</td>
<td>0.943</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>1.049</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NaCl: a=b=c=5.63
KCl: \(a = b = c = 6.26\)

\(\beta\)-FeOOH: Orthorhombic \(a = 3.08, b=12.5, c= 3.87\) angles = 90

\(\alpha\)-FeOOH: Orthorhombic \(a = 4.608, b= 9.956, c = 3.022\) angles = 90

\(\alpha\)-Fe\(_2\)O\(_3\): Hexagonal \(a = b = 5.038, c = 13.772, \alpha = \beta = 90, \gamma = 120\)

\(\gamma\)-Fe\(_2\)O\(_3\): cubic \(a = b = c = 8.342\) angles = 90

Fe\(_3\)O\(_4\): cubic \(a = b = c = 8.396\) angles = 90

Fe(OH)\(_3\): Orthorhombic \(a = 7.544, b = 7.560, c = 7.558\)