POLYMER MODIFICATION WITH MATRICES FOR MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY AND WITH APTAMERS FOR SURFACE-ENHANCED LASER DESORPTION/IONIZATION MASS SPECTROMETRY

By

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To my daughter, Elaine Rogers
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A resorcinol-formaldehyde (RF) polymer and matrix-embedded RF polymers were
developed to be used as substrates for improved matrix-assisted laser desorption/ionization mass
spectrometry (MALDI-MS) analysis and for selective analysis of biological samples.  

Matrix molecules can be embedded within this polymer, which renders excellent spectra
without the interference imposed by the matrix molecules for low molecular weight analytes. By
embedding the matrix molecules in this polymer more uniform spectra are obtained, solving the
prevalent problem associated with the traditional MALDI sample preparation methods.

The phenol groups on the polymer made it possible to attach aptamers selective to
reactive green 19 and cocaine onto it directly, and gave high specific affinity toward the reactive
green 19 and cocaine, respectively. The phenol groups on the polymer also made it possible to
attach carboxylic acid groups which could then be reacted with amine-modified aptamers. This
indirect aptamer-modified RF polymer has higher reaction efficiency than the direct aptamer-
modified RF polymer, yield more aptamers at higher density on the polymer for affinity capture
of analyte. This high specificity provided by the aptamers could make it a valuable tool to
specifically retain the biomarkers of diseases and could be used for screen test for diseases.
In the history of analytical chemistry, new technologies of analysis and instruments have been developed to better determine the compositions and structures of substances in order to identify them in the pursuit of understanding the world around us. The analytical methods that have been used include some very rudimentary but still widely used techniques such as titration, density, melting point, boiling point, flame test, and combustion, which are methods still taught to new chemistry students. As the tasks for chemical identification became more complicated and difficult, many new technologies were developed to peek into the microscopic world of chemistry. These technologies include atomic absorption, ultraviolet/visible (UV-vis) absorption, IR, fluorescence, flame, atomic emission, Raman, X-ray, X-ray crystallography, NMR, electrochemistry, gravimetric analysis, calorimetry, and thermogravimetric analysis (TGA), etc. Each of these more sophisticated technologies, based on a specific theory, utilizes specific chemical and/or physical properties of the substance, and requires a specific instrument to obtain specific information of the analyzed substance.1

A mass spectrometer is an instrument which was first invented at the beginning of the twentieth century, but took off in its application in the past thirty or forty years as a tool to identify various analytes. From gaseous molecules to volatile small organic molecules, then to involatile large organic molecules, and eventually to proteins, DNA, cells, and bacteria, the range of the analytes than can be analyzed has expanded since then. The high sensitivity, speed, specificity, and reproducibility of mass spectrometry (MS) have made it an irreplaceable tool for its application in protein analysis—including discovery, identification (i.e., peptide mapping and sequencing), and structural characterization.2
Introduction to Mass Spectrometry: 3-D and 2-D Quadrupole Ion Trap Mass Spectrometry

Introduction to Mass Spectrometry

The first mass spectrometer, built in 1907 by J.J. Thomson, was used to detect positive rays based on the mass-to-charge ratio (m/z) of the particles; this was the basis of modern mass spectrometry. The modern mass spectrometer is used to obtain the information on molecular mass according to the mass-to-charge ratio and even structures of molecules according to fragmentation patterns. The ionization device coupled to the mass analyzer has evolved because of the demand of an expanded variety of analytes. Electron ionization (EI) was the first used, followed by chemical ionization (CI), fast atom bombardment (FAB), electrospray ionization (ESI), and matrix-assisted laser desorption/ionization (MALDI).

Analyte molecules are ionized by addition or loss of an electron or a proton or other ion. The mass spectrum consists of the molecular-type ions (M+, [M+H]+, [M-H]-, ect.) and often fragment ions, the pattern of which may be unique for each molecule. The mass spectrometer was first used to identify elements and their isotopes. Soon after, petroleum chemists used the mass spectrometer to identify organic molecules. Now, the applications of the mass spectrometry have broadened to include biochemistry, explosives, fullerenes, toxics, and environmental pollutants.

A mass spectrometer is named according to the mass analyzer which is its determining component. During the process of the development of the mass spectrometer, many different types of mass analyzers have been invented, which include magnetic-sector, quadrupole mass-filter, 3-D quadrupole ion trap, 2-D quadrupole ion trap, and time-of-flight. Each of the mass analyzers has its forte and suitability for analysis of certain types of analytes and is chosen according to the task and budget.
Introduction to 3-D Quadrupole Ion Trap Mass Spectrometry

The quadrupole ion trap was invented by German physicist Wolfgang Paul and co-workers in the early 1950s. The quadrupole ion trap can be used to confine ions in a small volume using just high-frequency electric fields without a material container. This invention later earned Paul the 1989 Nobel Prize in Physics. Figure 1-1 illustrates a traditional 3-D quadrupole ion trap. This 3-D quadrupole ion trap has two hyperbolic end-cap electrodes with entrance and exit holes in the center of both end-caps. The hyperbolic ring electrode in-between the end-caps has a radius \( r_0 \). The distance between the two end-caps is \( 2z_0 \), where ideally, \( Z_0 = r_0 / \sqrt{2} \). Ions entering the trap are trapped at the center of the ion trap by a quadrupolar field which is created by a radio frequency (RF) waveform applied to the ring electrode when the two end-caps are grounded.

Equations 1-1 and 1-2 are derived from the Mathieu equation for a “stretched” ion trap, where \( e \) is the charge, \( U \) is the DC potential, typically kept at zero; \( V \) is the amplitude of the RF, and \( \Omega \) is the angular frequency of the applied RF. Solutions to these two equations define whether an ion with particular \( m/z \) will be stable inside the quadrupolar field according to their position in the Mathieu stability diagram (Figure 1-2).

\[
\begin{align*}
a_x &= -16eU/m(r_0^2 + 2z_0^2) \Omega^2 \\
q_z &= -8eV/m(r_0^2 + 2z_0^2) \Omega^2
\end{align*}
\]

The quadrupole ion trap confines ions in the small volume between the ring electrode and two endcap electrodes by appropriate oscillating (RF) electric fields. In modern ion trap, an AC waveform, termed the resonance ejection waveform, is applied across the endcaps. The frequency is typically set to be slightly less than half of the RF frequency, \( \Omega \). Since the oscillating frequency, \( \omega \), the ion motion in the \( z \) direction is given by \( \Omega B_z/2 \) (see Figure 2 for \( B_z \) values), this will correspond to a eject \( V \) value less than 0.908. When the amplitude of the
applied RF is linearly ramped up, a mass spectrum is obtained via the mass-selective instability scan. In this instability scan, ions are ejected from the trap with increasing $m/z$ with respect to time when they come into resonance with the resonance ejection waveform. Higher $m/z$ ions are still trapped; the limitation of mass range is due to an inability to reach the needed RF voltage levels required to eject higher $m/z$ ions out of the trap. In normal operation, for the instrument used for this research, the maximum $m/z$ detectable is 650. Lowering the AC resonance ejection frequency applied across the endcaps will allow ions to come into resonance earlier in the RF ramp, extending the mass range by lowering the $q_e$ according to the equation 1-3.11

$$ \frac{(m/z)_{\text{new}}}{(m/z)_{\text{old}}} = \frac{q_{eject \ new}}{q_{eject \ old}} $$

A quadrupole ion trap can be connected with external ion sources such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI). The range of ionization sources that can be used, in addition to its MS$^n$ capability, makes the quadrupole ion trap a very useful tool for compound identification.

**Introduction to 2-D Quadrupole Ion Trap Mass Spectrometry**

The 2-D ion trap, the linear variant of the 3-D ion trap are based on the four-rod 2-D quadrupole mass filter, omitting the quadrupolar-trapping field along one of the three spatial axes.8

The 2-D ion trap shown in Figure 1-3 12 is composed of three sections in which ions are mass selectively ejected radially through two slits in the two opposite rods in the center section. Ions are trapped in the $z$ direction by more positive DC voltages applied to the two end sections for axial trapping of positive ions (Figure 1-4).12 The ions are trapped radially by the RF applied in two phases to opposite rod pairs, as shown in Figure 1-4.12 A supplementary AC voltage is applied across the x-rod pair in two phases for isolation, collision-induced dissociation (CID), and resonant ejection of ions (Figure 1-4)12.
Ions confined within a linear trap can be mass-selectively ejected in a direction perpendicular to the central axis of the trap (radial ejection) or can be ejected along its axis (axial ejection). Having the detectors at both radial exit slits doubles the number of detected ions in radial ejection design. The axially-ejected ions can be introduced into a second mass analyzer such as a Fourier-transform ion cyclotron resonance (FT-ICR) analyzer to form a hybrid instrument.³

Relative to the well-established 3-D ion-trapping instruments with similar mass range, the 2-D linear trap with radial ejection has significantly higher trapping efficiency and increased ion capacity that improves the detection sensitivity at least 5 to 10-fold. This linear trap can conduct all the typical scan modes intrinsic to 3-D traps with comparable mass resolution but at higher overall scan rates.⁸

**Development of Ionization Methods for MS Analysis: from LD to MALDI and from MALDI to SELDI**

The ionization method used is determined by the type of the analyte and determines the characteristics of the mass spectrum obtained. The most common ionization methods include EI, CI, ESI, and MALDI.

Electron ionization was the first ionization method developed, and was used in the instrument invented by JJ Thompson. Electrons with an energy of about 70 eV collide with the vaporized analyte in vacuum (10⁻⁵-10⁻⁴ torr)³ to ionize the analyte molecules (Figure 1-5).¹³ The spectra obtained with EI are characterized by significant fragmentation of the molecular ions ([M]+). Later, when a softer ionization method was required for more fragile compounds chemical ionization (CI) was developed. With CI, electrons collide with a reagent gas present in large excess compare to the analyte to produce reagent ions which will later pass their charge to the analytes by collision without transferring an excess of energy (Figure 1-6).¹⁴ The spectra
obtained with CI are characterized by less fragmentation of the analyte and more abundant molecular-type ions ([M+H]+) than with EI. For these two ionization methods, the analytes have to be vaporized before they can be ionized, which is a problem for less volatile compounds. Typically, the higher the molecular weight, the less volatile the molecules. With EI and CI, it is difficult to ionize most of the large organic molecules and is nearly impossible to ionize macromolecules.

With increasing demand for analyzing large nonvolatile molecules, ionization methods that can ionize large, nonvolatile molecules were developed such as fast atom bombardment (FAB). With FAB, a beam of atoms bombard the analyte, which is dissolved in a low volatile liquid matrix, to produce the analyte ions (Figure 1-7).15 Since the analyte molecules are not vaporized before they are ionized, FAB can be used to ionize polar, ionic, thermally labile, and relatively high molecular weight compounds that are not suitable for normal EI/CI analysis. Depending upon whether the cation or the anion is of interest, positive-ion or negative-ion FAB/MS analyses can be performed. FAB can ionize molecules up to 2000–3000 Da.3

Laser desorption (LD) ionization was also developed to tackle the problem associated with less volatile large compounds. Introduced in the early 1960s, LD irradiated low-mass organic molecules with a high-intensity laser pulse to form ions that could be successfully mass analyzed. With LD, nonvolatile large molecules with the ability to absorb the energy from the laser beam can be ionized. Although LD expanded the range of the analyte that can be ionized, there were some limitations that came along with LD. The wavelength of the laser used in LD has to match the wavelength of the analyte molecule’s absorbance for the laser energy to be utilized for ionization. This may be difficult when a variety of analytes need to be analyzed. LD was also considered as an ionization method which is too energetic for labile analytes since laser
energy is imparted directly into the molecules, which may cause a large amount of thermal degradation and fragmentation.

First developed in 1980s, ESI was developed to ionize large and thermally labile molecules. By forcing a solution of the analyte through a small capillary held at high voltage, the solution sprays into tiny droplets with charges on each droplet. After the solvent is evaporated, ions from nonvolatile compounds with one or more charges are generated, as shown in Figure 1-8. The existence of multiple charges on high mass ions lowers the mass-to-charge ratio, which expands the mass range of the molecules can analyzed. As a soft ionization method, ESI makes it possible to investigate even intact noncovalent complexes. The invention is so important and revolutionary that the inventor, John B. Fenn of the Virginia Commonwealth University, shared with Koichi Tanaka of the Shimadzu Corp. half of the 2002 Nobel Prize in Chemistry for his invention of ESI.

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization method that rivals ESI for mass spectrometric analyses of biological macromolecules and the noncovalent complexes between peptides and metal ions, single-stranded DNA, amino acids, drugs and other peptides. In 1985, Michael Karas and Franz Hillenkamp published the laser desorption results of the mixture of aromatic amino acid which absorbs laser energy with aliphatic amino acid which does not absorb laser energy. In 1987, Karas and Hillenkamp successfully used a matrix in LD to ionize and analyze high molecular weight molecules. Matrices are organic molecules with aromatic groups that can absorb laser energy in UV. The most commonly used matrices are 2,5-dihydroxybenzoic acid (DHB), trans-3,5-dimethoxy-4-hydroxycinnamic acid or sinapic acid (SA), nicotinic acid (NA), trans-3-methoxy-4-hydroxycinnamic acid or ferulic acid (FA), 3-hydroxypicolinic acid (3HPA), 4-hydroxy-α-
cyanocinnamic acid (4HCCA), and (in the infrared) succinic acid and glycerol (Figure 1-9).

With MALDI, a low concentration of the analyte is mixed with a large excess of organic matrix to completely isolate analyte molecules from each other to homogenous 'solid solution'. The laser beam is focused onto the surface of the matrix-analyte solid solution. The matrix molecules absorb the laser energy at the incident laser wavelength and then pass that energy on to analyte molecules, producing a pulse of ions with each laser pulse. The clusters ejected from the surface consist of analyte molecules surrounded by matrix and salt ions. The matrix molecules evaporate away from the clusters to leave the free analyte in the gas-phase. The photo-excited matrix molecules are stabilized through proton transfer to the analyte. Cation attachment to the analyte is also encouraged during this process. It is in this way that the characteristic \([M+X]^+\) (\(X=\text{H, Na, K etc.}\)) analyte ions are formed. These ionization reactions take place in the desorbed matrix-analyte cloud just above the surface. The ions are then extracted into the mass spectrometer for analysis. MALDI allows a larger variety of analytes to be ionized by the same laser than LD.\(^{33}\) Figure 1-10\(^{14}\) is an illustration of ionization process of MALDI. The application of MALDI to biological macromolecules demonstrated by Koichi Tanaka \(^{34}\) led him to receive a quarter of the 2002 Nobel Prize for chemistry.\(^{17}\)

The matrix-to-analyte ratio ranges from 100 to 50,000.\(^{3}\) MALDI overcame the problem associated with the LD, but inevitably suffers from the interference from matrix molecules with analytes of low molecular weight. The excessive amount of matrix molecules poses interference to the interpretation of the mass spectra of low molecular weight analytes. Some effort has been put into developing matrix-free laser desorption/ionization methods to eliminate the interference from matrix. Desorption/ionization on porous silicon (DIOS) is one of the methods developed which utilizes the large surface area of an UV absorbing semiconductor treated electrochemically
to produce spectra without the interference of matrix clusters.\textsuperscript{35, 36, 37, 38} DIOS certainly has its success, but suffers from the relatively less than ideal sample-to-sample reproducibility, stability of targets, molecular weight limitation, low analyte signal intensity and is sensitive to how the samples are deposited on them-cracks can form with an inappropriate sample deposition method.

Since MALDI opened the gate for the MS analysis of macromolecules, biological samples have been subject to the MS analysis for disease biomarker discovery and clinical diagnostics. Disease biomarkers are the characteristic amount or the existence of a certain substance or substances.\textsuperscript{39} Biological samples taken directly from humans or animals such as blood, serum, plasma, urine, and cellular secretion products also contain extremely large numbers of components which are not of interest and can interfere with the MS analysis.\textsuperscript{40} These unwanted components include biological molecules and organic and inorganic salts which can make the MS analysis extremely difficult. Liquid chromatography (LC), high-pressure liquid chromatography (HPLC), membrane dialysis, centrifugation, immunoprecipitation, gel electrophoresis and other separation techniques have been used to separate and extract the target analyte from the biological sample prior to the MS analysis. Theses separation techniques are usually tedious and time-consuming and suffer loss of the analyte during the process, especially for the low abundant components.\textsuperscript{40}

Surface-enhanced laser desorption/ionization (SELDI) was introduced by Hutchens and Yip in 1993 in order to achieve better sample preparation.\textsuperscript{41} SELDI is based on MALDI but with the surface of the substrate modified to have a specific affinity toward certain types of molecules to achieve an on-probe, one-step clean-up procedure before the sample is analyzed. There are several companies which provide SELDI chips, such as Ciphergen Biosystems (Palo Alto, CA). Affinity capturing for enrichment and clean-up of analyte is borrowed from affinity
chromatography. Among them, immobilized metal affinity capture (IMAC), antibody and protein affinity capture, lectin affinity capture, inherent hydrophobic interaction bio-capture, and bio-molecular interaction capture are attractive ones, and have been used to retain biomolecules such as proteins and peptides.\textsuperscript{42} Figure 1-11 shows the biochemical and chemical modified affinity surfaces available from Ciphergen.\textsuperscript{42} The chemical modification of the substrate surface is well established. It retains a class of molecules which is useful for certain applications. Biochemically modified surfaces have specificity toward one specific molecule. Antibodies, DNA, enzymes, and receptors have been used in biochemical surface modification.

Aptamers are pieces of synthetic DNA or RNA oligonucleotides which recently been applied to SELDI affinity capturing.\textsuperscript{73} Aptamers are screened from a randomly generated population of DNA/RNA sequences for their ability to bind with desired molecular targets such as peptides with high specificity, and can be designed and synthesized easily with a DNA synthesizer. Aptamers have not yet been widely used for SELDI and are not available commercially.

**Introduction to Aptamers**

Aptamers have been employed in applications such as chromatography, capillary electrophoresis, biosensors, and signaling because of its ability of affinity capture small molecules, peptides, and proteins.\textsuperscript{43} Aptamers has also been used for mass spectrometry sample preparation in surface enhanced laser desorption/ionization (SELDI).

**Definition and Synthesis of Aptamers**

Aptamers are pieces of synthetic DNA or RNA with 3-D structures which provide the targets binding capability. The name “aptamer” is derived from the Latin word “aptus” which means “to fit”. Aptamers can be selected “to fit” small molecules, biopolymers, surfaces or even whole cells.\textsuperscript{43} Because of the high specific affinity, aptamers can be used as recognition
elements in heterogeneous assays to replace antibodies.\textsuperscript{44, 45} The selective binding affinity toward the target is due to specific interactions such as hydrogen bonding or association with the phosphate groups of the aptamer.\textsuperscript{46} The sequence-specific tertiary structure of the aptamer helps these interactions, which provides a rigid platform for the arrangement of functionalities of the aptamer. The 3-D structures have been discovered to include the stem-loop/bulge (Figures 1-12 and 1-14),\textsuperscript{47, 48} the helix, the hairpin (Figure 1-13),\textsuperscript{47} the pseudoknot (Figure 1-14),\textsuperscript{49} and the G-quartet structure (Figure 1-15).\textsuperscript{50} Under certain condition such as the buffer with certain pH, certain components, and concentration; aptamer can form 3-D configuration which retains a specific protein or molecule which can fit in this 3-D configuration. Because of its affinity capture capability, aptamers have been used in chromatography for separation and purification of mixtures.\textsuperscript{48, 51}

An aptamer for a particular target is discovered and produced by the method called systematic evolution of ligands by exponential enrichment (SELEX), which utilizes in vitro selection and amplification procedures to isolate aptamers for any small molecule or protein target.\textsuperscript{52, 53} This automatic method was developed independently by Joyce, Szostak, and Gold.\textsuperscript{52, 54, 55} First, a library of oligonucleotide molecules with the size of $10^{15} \sim 10^{18}$ is generated chemically in a standard oligonucleotide synthesizer.\textsuperscript{51} The greater the number of randomized nucleotide positions, the more complex a library is and the more likely the success of finding the molecules that interact with a target.\textsuperscript{44} Each member in a library has a unique sequence which is designed to contain a contiguous randomized region flanked by two fixed sequence regions. Technological advances already have made it possible to eliminate the requirement for the fixed regions in random sequence libraries used for the SELEX process, thereby producing short aptamer sequences. A mixture of phosphoramidites containing all four building blocks: A, G, C,
and T is delivered to the synthesizer to synthesize each nucleotide position in the contiguous random region. The ratios of the four phosphoramidites in the mixture are adjusted based on the coupling efficiencies of individual monomers to obtain an unbiased library with equal representation of all four nucleotides. Because aptamers are identified from DNA as well as from RNA libraries, chemically synthesized DNA libraries are enzymatically converted to RNA libraries.  

The screening process is the next step, in which a random sequence oligonucleotide (RNA or DNA) library is incubated with a target of interest in a buffer of choice at a given temperature (see Figure 1-16). A very small fraction of individual sequences tend to interact with the target, and these sequences are separated from the rest of the library by any one of the physical separation techniques. Typically, nitrocellulose filter partitioning is used with protein targets that are retained on the nitrocellulose. Small molecular targets are generally immobilized on a solid support to generate an affinity matrix in which case, sequences that do not interact with the target on the solid support can be removed easily by a simple washing step. The mixture of aptamer candidates recovered from the target represents a mixture of sequences containing both high- and low-affinity binding molecules to the target. The sequences bound to the target are isolated and amplified to obtain an enriched library to be used for the selection/amplification cycle.

The DNA aptamer candidate mixture is amplified directly by the polymerase chain reaction (PCR), while RNA sequences are amplified by PCR after being converted from DNA by reverse transcription (RT). The single-stranded DNA (ssDNA) population obtained by strand separation of PCR products is incubated with a fresh sample of the target for the next round of selection. The RNA population is obtained by in vitro transcription. Several iterations of the
selection process are carried out under increasingly stringent conditions to enrich the high-affinity sequences and eliminate the low-affinity binder. The enrichment efficiency of high-affinity binders is controlled by the stringency of selection at each round. Analysis of enriching populations against the target is carried out to determine the progress of the enrichment of high-affinity binders.44 The number of cycles required for aptamer identification is usually dependent on the degree of stringency imposed at each round as well as on the nature of the target. For most targets, affinity enrichment is reached within 8–15 cycles. In general, one cycle of SELEX takes two days. The enriched library is cloned and sequenced to obtain the sequence information of each member until the chosen sequences dominate the population.55 A typical SELEX experiment may take approximately 2–3 months including cloning and sequencing.58

Aptamers that come out of a SELEX experiment are full-length sequences which are generally 70–80 nucleotides long, and can be truncated to eliminate nucleotide stretches that are not important for direct interaction with a target or for folding into the structure that facilitates target binding. The truncation of aptamers to the minimal target-binding domain has been successfully carried out to obtain functional aptamers less than 40 nucleotides long.58, 59, 60, 61, 62, 63 Once the sequence is identified, an aptamer is produced by chemical synthesis.

Aptamers have remarkable specificity and can discriminate targets on the basis of subtle structural differences such as the presence or absence of a methyl group64, 65 or a hydroxyl group66, 67 and the D- vs. L-enantiomer of the target.66, 68 Because of the selective demand in the SELEX process that eliminates sequences that bind closely related analogs of the target, sometimes the degree of specificity of aptamers is better than that of antibodies.64 Practically, elimination of the sequences that bind closely related analogs of the target is achieved by the process called "counter-SELEX" that effectively discards ligands that have an ability to bind the
target as well as closely related structural analogs of the target. During counter-SELEX selection, the population of aptamers bound to the target is subjected to affinity elution with structural analogs and the sequences eluted are discarded.

The counter-SELEX strategy is a valuable tool in identifying aptamers aimed at a specific target in a complex mixture, even without prior knowledge of the target. For example; in the search for aptamers that bind to an "epitope" only present on the surface of cancer cells but not in healthy cells, the cells from healthy tissue are used to remove sequences that bind to the background that does not contain the epitope of interest before the library is challenged with cancer cells.

**The Advantages of Using Aptamers vs. Antibody and the Applications in Bioanalysis**

Both antibodies and aptamers are utilized in bioanalysis based on their molecular recognition capability. Antibodies are the most popular class of molecules providing molecular recognition. They have been used in SELDI because of its high specificity and selective affinity towards a specific target.

Antibodies started to get attention since the 1950s and became very popular in the 1970s. Although antibodies have the advantages of having a very high selective affinity, they also have several limitations.

First, antibody generation involves untidy cell cultures and lab animals since antibodies must be selected and produced in a living organism. Antibodies of non-human origin have implications in diagnostic applications; thus, the use of antibodies in therapeutic applications is limited since the generation of hybridomas is restricted to rats and mice. Heterophilic antibodies (human antibodies that recognize antibodies of non-human origin) that exist in humans might lead to false-positive results because a capture antibody can be linked with a detector antibody of non-human origin in the absence of the specific analyte.
Second, stocks of antibody-producing cells need to be stored at multiple sites to prevent the complete loss of the cell lines because of the possibility of accidental losses or the death of cell lines. Typically, high yields of monoclonal antibodies are obtained by growing the hybridomas in the peritoneal cavities of animals and purifying the antibody from ascites (abdominal dropsy) fluid. Some hybridomas are difficult to grow in vivo, thus restricting this route of antibody production.44

Third, molecules that are not well tolerated by animals, such as toxins have difficulty being employed to produce antibodies in living organisms.44 Furthermore, it is difficult to raise antibodies against inherently less immunogenic molecules.44

Fourth, the identification and production of monoclonal antibodies are laborious and could become very expensive for searches of rare antibodies that require screening of a large number of colonies.44

Fifth, the consistent performance of the same antibody from batch to batch is poor; thus, immunoassays need to be optimized with each new batch of antibodies.44

Sixth, since the antibodies are produced in vivo and subject to in vivo variations, it is not practical to identify antibodies that could recognize targets under conditions other than physiological conditions.44

The ability of aptamers to bind to various targets makes aptamers applicable as biosensors, imaging probes, MALDI targets, and drugs.51 First, the properties of aptamers can be changed on demand because aptamers are identified through an in vitro process that does not depend on in vivo conditions. Furthermore, selection conditions can be designed to obtain aptamers with properties desirable for in vitro diagnostics.44
Second, as opposed to antibodies, toxins as well as molecules that do not elicit a good immune response can be used to generate high-affinity aptamers because animals and cells are not involved in aptamer identification.\textsuperscript{44}

Third, aptamers are stable during long-term storage and can be transported at ambient temperature. Aptamers can undergo reversible denaturation process and easily recover from exposure to undesirable conditions within minutes. The in vitro selection process for aptamers can be carried out under conditions akin to those used in the assay for which the aptamer is being developed. The aptamer will maintain its structure and function in the final assay and not fall apart as antibodies do.\textsuperscript{51}

Fourth, aptamers with any sequences that can recognize any class of target molecules with high affinity and specificity can be discovered with SELEX process. Once the sequence of a particular DNA aptamer is known, it is easy and inexpensive to synthesis the aptamer in a DNA synthesizer with high accuracy and reproducibility. They are then purified under denaturing conditions to achieve very a high degree of purity with little or no batch-to-batch variation in aptamer production.\textsuperscript{44, 51}

Fifth, additional chemistries can be added on without a loss in function because aptamers are chemically synthesized, which gives aptamers superiority over antibodies. When chemical groups are attached to the ends of the aptamers, their life span in the bloodstream increases, they can be targeted to particular locations, or they can be immobilized onto a surface. Immobilizing aptamers without potential loss of function makes it superior to antibodies.\textsuperscript{51} Reporter molecules such as fluorescein and biotin can be attached to aptamers at exact locations designed by the user. Functional groups that allow subsequent derivatization of aptamers with other molecules can also be attached during the chemical synthesis of aptamers.\textsuperscript{44}
Sixth, aptamers can also be used for the development of sandwich assays for small molecules or modified into a molecular-beacon format (a DNA strand with a fluorescent tag and a quencher) for reagentless assays, which is beyond the capability of antibodies.\textsuperscript{51}

Seventh, aptamers have smaller molecular weight, which offer advantages such as ease to penetrate tissue, shortened residence time in blood, and a smaller footprint when being attached to a surface to get a higher binding density.\textsuperscript{51}

Because of the advantages the aptamers posses over antibodies, the aptamers can be used to replace antibodies to retain molecules, although the recognition ability of aptamers is not as high as antibodies.\textsuperscript{51}

A lot of effort has been put into research to explore the usability of aptamers in analytical chemistry. SomaLogic (Boulder, CO) is developing aptamer proteomic chips as diagnostic tools that screen for biomarkers in serum. Ellington’s group from Department of Molecular Biology, Massachusetts General Hospital, Boston, has demonstrated that aptamers can be immobilized on beads, introduced onto a sensor array, and used for the detection and quantitation of proteins.\textsuperscript{71} Kennedy’s group from University of Florida showed that DNA aptamers immobilized on a chromatographic support can selectively bind and separate adenosine monophosphate (AMP), cyclic-AMP, adenosine diphosphate, adenosine triphosphate, NAD\textsuperscript{+}, and adenosine from mixtures as complex as tissue extracts.\textsuperscript{72} McGown from Duke University successfully used a thrombin-binding DNA aptamer for affinity capture for MALDI-MS.\textsuperscript{73} In her work, she attached aptamer pieces onto a treated fused-silica glass surface and then used the aptamer-attached glass to capture the thrombin from a protein mixture. The result showed that the thrombin-binding aptamer has high affinity toward the thrombin.
Overview of Dissertation

The importance of developing a new MALDI substrate and the suitability of the RF polymer for MALDI-MS analysis was emphasized at the beginning of the dissertation.

A new RF polymer and matrix-derived RF polymers have been developed and characterized as discussed in Chapter 2 for elimination of the background noises from matrix molecules used in MALDI-MS analysis and for more even distribution of signal intensity of analyte across the sample spot.

Surface modification of the RF polymer with carboxylate groups and then with amine-modified aptamers have been performed and characterized as discussed in Chapter 3 for attachment of functional groups and then attachment of functionally-modified aptamers onto the RF polymer.

With the affinity capture capability of this SELDI-MS substrate, it is possible to perform an on-probe one-step clean-up for the specific small molecules such as reactive green 19 and cocaine before the MS analysis of a biological sample. The polymer and modification procedures are capable of being applied later on disease biomarkers. This is crucial for large-scale cancer screening to be accessible for the vast majority of the population at lower cost and higher accuracy.
Figure 1-1. Scheme of parts of the 3-D quadrupole ion trap. [Adopted from March, R.E. An introduction to quadrupole ion trap mass spectrometry Journal of Mass Spectrometry 1997, 32, 351]
Figure 1-2. Stability diagram in \((a_z, q_z)\) space for the region of simultaneous stability in both the \(r\)- and \(z\)-directions near the origin for the three-dimensional quadrupole ion trap; the iso-\(\beta_r\) and iso-\(\beta_z\) lines are shown in the diagram. The \(q_z\)-axis intersects the \(\beta_z=1\) boundary at \(q_z=0.908\), which corresponds to \(q_{\text{max}}\) in the mass-selective instability mode. [Adopted from March, R.E. An introduction to quadrupole ion trap mass spectrometry *Journal of Mass Spectrometry* 1997, 32, 351]
Figure 1-3. Basic design of the two-dimensional linear ion trap. [Adopted from Schwartz, J.C.; Senko, M.W. A two-dimensional quadrupole ion trap mass spectrometer *Journal of the American Society of Mass Spectrometry* **2002**, *13*, 659]
Figure 1-4. Scheme for application of DC, RF trapping, and AC excitation voltages necessary for operation of the 2-D ion trap. [Adopted from Schwartz, J.C.; Sento, M.W. A two-dimensional quadrupole ion trap mass spectrometer *Journal of the American Society of Mass Spectrometry* **2002**, *13*, 659]
Figure 1-5. Electron ionization schematic. [Adapted from http://www.noble.org/plantbio/MS/iontech.ei.html]
Figure 1-6. Chemical ionization schematic. [Adapted from http://www.noble.org/plantbio/MS/iontech.ci.html]
Figure 1-7. Illustration of FAB. [Adapted from http://www.chm.bris.ac.uk/ms/theory/fab-ionisation.html]
Figure 1-8. Electrospray ionization schematic. [Adapted from http://www.noble.org/plantbio/MS/iontech.esi.html]
Figure 1-9. Most commonly used MALDI matrices
Figure 1-10. A schematic diagram of the mechanism of MALDI. [Adapted from http://www.noble.org/plantbio/MS/iontech.ci.html]
Figure 1-11. The chemical and biochemical surfaces available for SELDI from Ciphergen provides. [Adopted from Tang, N.; Tornatore, P.; Weinberger, S.R. Current developments in SELDI affinity technology *Mass Spectrometry Reviews* 2004, 23, 34]
Figure 1-12. The bulge and stem structure of aptamers. [Adopted from Biroccio, A.; Hamm, J.; Incitti, I.; Francesco, R.D.; Tomei, L. Selection of RNA aptamers that are specific and high-affinity ligands of the hepatitis C virus RNA-dependent RNA polymerase. *Journal of Virology* 2002, 76, 3688]
Figure 1-13. The hairpin structure of aptamer. [Adopted from Biroccio, A.; Hamm, J.; Incitti, I.; Francesco, R.D.; Tomei, L. Selection of RNA aptamers that are specific and high-affinity ligands of the hepatitis C virus RNA-dependent RNA polymerase *Journal of Virology* **2002**, *76*, 3688]
Figure 1-14. The pseduknot structure of aptamer. [Adopted from Tuerk, C.; MacDougal, S.; Gold, L. RNA pseudoknots that inhibit human-immunodeficiency-virus type-1 reverse-transcriptase Proceedings of the National Academy of Sciences of the United States of America 1992, 89, 6988]
Figure 1-15. The G-quartet structure of an aptamer. [Adopted from Wang, K.Y.; McCurdy, S.; Shea, R.G.; Swaminathan, S.; Bolton, P.H. A DNA aptamer which binds to and inhibits thrombin exhibits a new structural motif for DNA Biochemistry 1993, 32, 1899]
Figure 1-16. Generalized scheme indicating the key steps in the SELEX process. [Adopted from Klug, S.J.; Famulok, M. All you wanted to know about SELEX Molecular Biology Reports 1994, 20, 97]
CHAPTER 2
MATRIX-DERIVED RESORCINOL-FORMALDEHYDE POLYMERS: SYNTHESIS VIA SOL-GEL METHOD AND USAGE IN MALDI-MS SAMPLE PREPARATION

Since the invention of MALDI-MS, it has been used to analyze macro-biomolecules such as peptides mixtures, proteins, oligosaccharides, enzymatic protein digests, underivatized DNA, and oligomers. Although MALDI-MS is a powerful analysis method, it has problems associated with the sample preparation methods. Identifying and solving the problems can improve the quality of MALDI-MS spectra.

Problems Associated with MALDI-MS Sample Preparation Methods

As a widely used ionization method for mass spectrometric analysis for macromolecules, MALDI has shortcomings associated with the methodology of sample preparation. These problems include background interference from matrix molecules when the molecular weight of the analyte is less than 2-3 times the molecular weight of the matrix molecule and poor signal uniformity of the signal intensity of analyte across the sample from the inhomogeneous distribution of the matrix-analyte co-crystals. These problems can either interfere with the interpretation of MS spectra of low-mass analytes or require significant effort to locate the “sweet spot” – both of which routine analysis with MALDI-MS more difficult. Therefore, to eliminate the interference from matrix molecules and at the same time to increase the uniformity of the analyte signals intensities in MS spectrum, development of a polymeric substrate which has the physical properties amenable to MALDI-MS analysis and has the chemical properties to incorporate matrix molecules into its structure is highly desirable.

Background Interference from Matrix Molecules in MALDI-MS

MALDI ionization coupled with mass spectrometry made the analysis of nonvolatile molecules possible. In MALDI, an excessive amount of matrix molecules, with the matrix-to-analyte ratio typically in the range of 100 to 50,000, is mixed with analyte in order to ionize the
analyte particles. The excessive matrix molecules are not anchored to any support, and, inevitably, the matrix molecules will be released and ionized upon ablation of the laser beam, and appear in the mass spectrum together with the analyte. Signals produced by the matrix molecules present in the mass spectrum along with the signals from analyte pose interference in analysis of low molecular weight analyte.

Much effort has been devoted into finding the mechanism of MALDI which can lead to the development of matrix suppression methods. Competition model, in which competition between matrix and analyte for free protons based on the proton affinity, is one of them. According to this model, matrix suppression takes place when adjusting the matrix-to-analyte ratio for every protonated matrix molecule to interact with at least one analyte with higher proton affinity than the matrix molecule during desorption. In another proposed mechanism, analyte molecules are ionized by non-ionic matrix precursor species which are vibrationally and electronically excited matrix molecules. Knochenmuss and Dubois proposed a model combining the previous two models. In this model excited, but not ionized, matrix molecules are the common precursor for all subsequent ion products and the simultaneous neighboring presence of two such excitations is required for ionization of each analyte molecule. This model explained matrix suppression phenomena observed in a medium matrix-to-analyte ratio with relatively low analyte signal intensity and less matrix suppression in a high matrix-to-analyte ratio. According to this mechanism, suppression of the matrix signals has to be compromised to get high enough analyte signal intensity, or vice versa, when only the matrix-to-analyte ratio is involved.

Modifications other than adjusting the matrix-to-analyte ratio in MALDI sample preparation have been made in order to reduce the matrix interference in mass spectrometric analyses. These modifications include adding iodine to the matrix to suppress the signals from
the matrix-related ions and increase the signals from analyte ions,\textsuperscript{84} using alkali
dihydroxybenzoic acid salts as cationizing agents to suppress the DHB related ions,\textsuperscript{85} mixing
graphite powder with glycerol to reduce the interference,\textsuperscript{86, 90} and using nitrocellulose as a
substrate together with matrix to eliminate the matrix signals.\textsuperscript{88} In each case the modification
can reduce the signal intensities from matrix molecules but cannot completely eliminate them.
In all those methods, the matrix molecules are not covalently attached to any support but merely
interact with the support by dipole-dipole interaction and van der Waals force; which are at the
magnitude of one-thousandth and one-ten thousandth of the strength of the covalent bond,
respectively. Therefore, are easy to be ablated and ionized when the energy of the laser beam
shot on the target is much higher than the energy required to break these interactions.

In an effort to fundamentally solve the matrix related problem, Lin and Chen incorporated
matrix molecules into the gel formed from the tetraethoxysilane (TEOS) precursor via sol-gel
method.\textsuperscript{75} Total elimination of the matrix molecule signals was observed when small proteins,
peptides, amino acids, and small organics were analyzed. In the experiment, the matrix (DHB)
was mixed with the monomer precursor to form the sol-gel solution and was claimed to become
part of the polymer structure after the solution is polymerized. The mechanism of the
polymerization reaction was proposed as shown in Figure 2-1. The hydroxyl groups on the
matrix molecules can form covalent bonds with the hydroxyl groups in the silicic acid and thus
covalently incorporated into the TEOS polymer as shown in Figure 2-2.

Since the energy required to break the covalent bond between the matrix molecule and the
polymer is greater than the laser energy used in MALDI, trapping the matrix molecules
covalently into the polymeric structure make it possible to totally eliminate the matrix ions’
background interferences in a MS spectrum. One problem associated with the TEOS gel, which
was not mentioned in the paper, is that cracks formed when an aqueous solution was applied onto it since the structure of the gel is not strong and can be easily broken. Developing a different matrix-embedded polymer which has better physical properties than the TEOS polymer is valuable for the polymeric substrate to be useful for MALDI-MS analysis with analytes in aqueous solution.

The Uneven Distribution of Analyte Molecules with the Traditional MALDI-MS Sample Preparation Methods

There have been many investigations on the effect of MALDI sample preparation methods on the quality of mass spectrometric analyses. MALDI-MS analyses results have strong dependence on the sample preparation method. Every single choice such as the solvent, matrix, and pH in sample preparation procedure can affect the outcome of the MALDI measurements.\textsuperscript{89, 90, 91, 92, 93, 94, 95, 96} The different combination of matrices, concentration of matrix, and solvent produce different distribution of matrix molecules across the sample as shown in Figure 2-3.\textsuperscript{97} The problem which concerns the mass spectrometry analysts the most is the poor spot-to-spot and sample-to-sample reproducibility caused by inhomogeneous distribution of the matrix-analyte co-crystal.\textsuperscript{93}

The methods used for the application of the analyte sample includes the rudimentary “dried-droplet” method which is to deposit matrix and sample mixture on the target with the matrix-to-analyte ratio as $10^3$ to $10^5$ and let it dry.\textsuperscript{98} This method is also called the “air dried” method.\textsuperscript{99} The formation of small co-crystals which contain matrix molecules and analyte molecules in the dried-droplet method is crucial for the ionization of the analyte molecules. The crystals formed in the sample prepared from dried-droplet method are not evenly distributed throughout the sample plate. The analyte-matrix co-crystals are found mostly at the edge of the droplet when DHB is used as matrix\textsuperscript{100, 101} as shown in Figure 2-4\textsuperscript{102} according to the partitioning
process when solvent slowly evaporates. When the solvent slowly evaporates the analyte molecules are excluded from the matrix crystal. An analyst would, therefore, to find “sweet spot” by scanning the sample surface.

A “thin-layer” sample preparation method has been developed based on the dried-droplet method using a volatile solvent to create homogeneous matrix microcrystals because quick evaporation of the solvent can minimize the partition process which occurs under the slow evaporation process. A drop of matrix solution in volatile solvent was deposited onto a target plate and was allowed to spread and dry quickly to form a thin layer of homogeneous matrix microcrystals. Then a drop of analyte solution was deposited on the top of the matrix layer. The spectra obtained from the thin-layer method produce more uniformed distribution of the analyte-matrix crystals than from the dry droplet method, therefore more uniformed analyte signal intensity across the sample (Figure 2-4).

“Sandwich” sample preparation method is a combination of the thin layer and the dried-droplet method. It is called sandwich because sample solution is applied on one layer of matrix crystals, then another matrix solution is added on the sample spot. This method has better mapping results than the dried-droplet method.

To utilize the quick evaporation of the solvent further, electrospray sample preparation method was used to further improve homogeneity of the components distribution in MALDI-ToF MS than the use of volatile solvents and/or rapid evaporation under vacuum or heating and the small and evenly distributed droplets can. This method produced much better spot-to-spot and sample-to-sample reproducibility in the mass spectra than dried-droplet method.

Since the selection of the matrix is very importance in some cases, experiments using more than one matrix for the sample preparation have been also conducted. These improvements are
made on a case-by-case basis. Limiting the size of the droplet is another technique that has been tested to improve the MALDI-MS analysis quality.103

Over all, there is not a universal sample preparation method that produces high quality mass spectra for variety of analytes. Mass spectra quality can be improved by modifications made to the sample preparation methods. Elimination of the poor spot-to-spot reproducibility of the analyte molecules (the uneven distribution of analyte across the sample) is impossible with the efforts have been done, because this problem is related to the process of the crystallization. In the matrix-embedded polymeric MALDI-MS substrate, the matrix molecules are evenly distributed throughout the polymer structure on the molecular level and cannot be changed during the sample preparation process and no analyte-matrix co-crystals are formed, thus, the matrix-embedded polymer has the capability of solving this problem.

**Introduction to the RF Polymer**

According to the discussion above, a matrix-embedded polymeric substrate is the solution to the background noise from matrix molecules in mass spectra and the poor spot-to-spot and sample-to-sample reproducibility of MALDI mass spectra. Matrix-derived TEOS polymer was used by Lin and Chen75 and was synthesized and tested in our lab. The matrix-derived TEOS polymer was able to significantly lower the matrix signals when using MALDI-MS. Although the TEOS gel has the advantage of lowering the chemical noise from matrix molecules, the structure of the gel is fragile and is easy to break apart, cracks when aqueous solutions were deposited on it. This shortcoming made it a less than ideal substrate for MALDI-MS since aqueous solutions are usually used and made it impossible for easy manipulation and further chemical modification. Resorcinol-formaldehyde (RF) polymer was finally chosen because of its tough physical structure and abundant phenol groups on it which render the capability of
being chemically modified. Resorcinol-formaldehyde resins have been used as adhesion promoters to increase adhesion of rubber to fabric or metal.

RF sol-gels were first synthesized by Pekala and co-workers according to a hydrolysis-condensation reaction mechanism (Figure 2-5). Figure 2-6 shows the base catalyzed polymerization reaction of RF polymer. The “R” in the RF polymer is referred to “resorcinol”, the “F” in the RF polymer is referred to “formaldehyde”. A patent was filed by Pekala on the synthesis of RF polymer. Resorcinol-formaldehyde resins have been used as adhesion promoters to increase adhesion of rubber to fabric or metal.

A lot of research has been conducted since then on the effect of different reaction conditions on the final structures of the polymers which affect the physical, chemical, and electrochemical properties of RF aerogels and xerogels. Many patents have been filed and approved for RF polymers with different reaction conditions. Those reaction conditions include the ratio between the resorcinol and formaldehyde, the solvent and catalyst, the initial pH of the reaction, the ratio between the catalyst and the resorcinol, the temperature of the reaction, and initial ratio between the reactants and the solvent. With these variables there are tremendous possibilities of combinations of the conditions which make it a very versatile reaction that can be tailored for the desired properties.

The RF polymers are categorized according to the solvent used. The gels with water solvent are called hydrogels or aquagels; the gels with organic solvents are called lyogels. The initial reactants and the solvent ratios of the reaction affect the final density of the gel’s size. Using higher concentrations of solvent can result in “dilute effect” which results in increased particle size. Using the higher concentrations of the reactants can result in higher density of the formation of the RF crosslinked clusters.
The most commonly used stoichiometric resorcinol / formaldehyde (R/F) molar ratio is 1:2. Using more formaldehyde than twice the amount of the resorcinol as the starting material would cause a “dilute effect”—the effect caused by higher solvent concentration.

The most commonly used catalyst is sodium carbonate. The molar resorcinol-to-catalyst (R/C) ratio is usually between 50 and 300, or can be as high as 1500. The lower the R/C ratio, the smaller the polymer particles and the higher density of the gels. In some cases acids such as HClO₄ or HNO₃ were used as catalyst, respectively, with reduced gelation time. Using an acid catalyst combined with low concentration of reactants can result in small, smooth, fractal aggregates of gel particles.

The pH should be controlled between 5.4 and 7.6 since high pH can hinder the polymerization-condensation reaction and reactants precipitate at low pH. Diluted acid such as HNO₃, HClO₄ and bases such as NH₄OH can be used to adjust the initial pH of the reaction solution.

**Systematic Investigation in Preparation of the RF and Matrix-Derived RF Polymers**

To address the problems facing analysts working on MALDI-MS, RF polymers were synthesized to be suitable as substrate for MADLI-MS. The RF polymers developed were wet gels without any further drying processes. These polymers are made via the quick and easy sol-gel method which makes it easy to be customized with any desired composition, on any surface, and any shape and size. The solutions can be stored in freezer for about 6 months. The polymer has a very strong structure due to the 1,3 bonding position on the aromatic rings in the polymer. The polymer did not crack upon the deposition of aqueous solution and can only be broken by a severe impact.

Usually the resorcinol formaldehyde ratio is 1:2, both acid and base were used as a catalyst with different molar ratio of resorcinol/catalyst (R/C). Water, acetone, methanol,
ethanol, n-propanol, and iso-propanol can all be used as the solvent. The catalyst used, ratio of R/C, the solvent, and concentration of the starting materials determine the final structure and properties of the gel. Eventually, acid (HCl) was chosen as the catalyst to provide H⁺ ions which are necessary for MALDI; acetone was chosen as solvent for quick polymerization; different matrices were used to investigate the ionization capability. The matrix molecules used for MALDI with similar structure as the RF polymer can be embedded into the polymer via covalent bonding.

The ratio of the resorcinol and formaldehyde is chosen as 1:2 since the ratio of the resorcinol and formaldehyde in the structure is 1:2. Water, alcohol, and acetone have all been used in the literature and suitable as the solvent of the RF polymerization reaction. Different solvents have been tested and eventually acetone was chosen as the solvent throughout the rest of the synthesis. With acetone as solvent, the temperature of the curing stage can be lowered significantly (from 80 °C to 40 °C) with reasonable gelation time. The initial concentration of the reactants was chosen as (R+F)/solvent 30% w/w. The sodium carbonate was first chosen as the catalyst because it is the most commonly used in the previous literature. The higher the ratio, the smaller the polymer particles and the higher density of the gel. The R/C ratio can be as high as 200:1 was chosen as the R/C ratio. Since an acidic polymer can provide H⁺ which is necessary in MALDI to ionize analyte, acids were tested. HClO₄ was used as first acid catalyst because it was used in the literature. Later, HCl was used throughout the rest of the synthesizing because it is a commonly used acid in lab, although there is no evidence in the literature about using HCl as catalyst in RF polymer synthesis.

The initial synthesis of the RF polymer was tested with the following procedure: 4.00 g Resorcinol (0.036 mol), 5.00 mL formaldehyde (0.072 mol), 12.00 mL acetone, and 0.0078 g
Na₂CO₃ (0.036 mmol) were added to the flask which was sealed and immersed in a 45°C water bath. The formaldehyde-to-resorcinol ratio is 2:1 and the resorcinol-to-Catalyst ratio is 1000:1. Every half hour several drops of solution were taken out of the flask and deposited on a glass slide. The solutions taken out at one hour and after one hour all successfully polymerized. Thus, the reaction time was then determined as one hour. The solution is stored in a vial in the freezer with -20°C. The solution can be used for six months after synthesis. This RF polymer was given the serial number as RF-1.

There were thirty nine experiments that have been conducted in an effort to systematically investigate the properties of the RF polymerization process. The tests were conducted using a combination of different solvents such as acetone, ethanol, and water; different resorcinol/catalyst ratios; different catalysts; different matrices with varying concentration; and addition of different doping agents. Every RF solution was named with “RF” followed by the serial number assigned from one to thirty nine.

Thirteen RF polymers have been synthesized with Na₂CO₃ as a catalyst and acetone as solvent as shown in Table 2-1. These polymers have a different resorcinol-to-catalyst ratio, with the addition of different matrices with varying concentration and the addition of doping agents in order to bring functional groups into the polymer.

The resorcinol-to-catalyst was changed to 500 and 100 which are assigned the serial numbers RF-2 and RF-39, respectively. These two polymers, together with the RF-1 polymer, all polymerized. After the successful synthesis of the RF-1 and RF-2 polymers, RF-3 polymer was synthesized with 1000 ppm of DHB which also polymerized as expected. The RF-20 polymer was able to polymerize when the concentration of the DHB was raised to 40,000 ppm with the high catalyst concentration (resorcinol-to-catalyst ratio as 100).
After DHB was successfully incorporated into the RF polymers, CHCA was tested for incorporation into the RF polymers with different concentration, different resorcinol-to-catalyst ratio, and different concentration of 4-(imidazole-1-yl)phenole with or without being cued with HCl which were assigned the serial numbers from RF-27 to RF-35. RF-29, RF-30, and RF-31 polymerized, while others did not. The R/C can be as high as 1000 for the sol to polymerize as long as there is no imidazole added even when there is matrix added. When there is imidazole added, the R/C can not be higher than 50. The concentration of the matrix can be as high as 10,000 ppm when CHCA was used.

Ethanol was tested as a solvent for RF-4 with 1000 resorcinol-to-catalyst ration and 1000 ppm DHB, and RF-5 with 500 resorcinol-to-catalyst ration and 1000 ppm DHB. Both of the sol crystallized instead of polymerized (Table 2-2).

Nine polymers (RF-6 to RF-12, RF 15, and RF 19) were tested in the water as solvent with varying concentration of Na$_2$CO$_3$ as catalyst, varying concentration of DHB, and addition of Fe$_2$Cl$_3$·H$_2$O in order to add metal chelating capability as shown in Table 2-3. All of these polymers polymerized with varying reaction time.

HClO$_4$ was the first acid catalyst used in the synthesis of RF-12 polymer with water as solvent. The necessity to test an acid catalyst is based on the need of the protons in order to ionize analyte molecules in MALDI. HClO$_4$ is documented as an acid catalyst for the synthesis of RF polymer, thus was chosen. Since storing HClO$_4$ requiring extra caution, HCl was test as catalyst in the synthesis of RF polymer. Although there is no evidence in the literature about using HCl as a catalyst for RF synthesis, the experiment was a success.

There are thirteen polymers (RF-14, RF-16 to RF-18, RF-21 to RF 26, and RF-36 to RF 38) that were synthesized with HCl as catalyst for its proton; with acetone as solvent for the fast
evaporation during the gel process; and with different matrices and varying concentration, with addition of imidazole, EDTA, and vinylimidazole; as shown in Table 2-4. Only seven of them polymerized (RF-14, RF-16, RF-21 to RF23, RF-25, and RF-38).

Among the thirty nine tests twenty four of them were able to polymerize. Nineteen of the polymerized solutions contain matrix. For each matrix-containing polymer, mass spectrometry was conducted with the sol-gel solution deposited on a stainless steel probe. After the solution was polymerized, the polymer was taped onto the stainless steel with double-sided tap, and then 100 ppm spiperone in methanol was deposited on the polymer. After the spiperone solution dried the sample was analyzed in a custom built MALDI ion trap mass spectrometer.

Among the twenty four matrix-embedded RF polymers, only the CHCA-embedded RF polymers produced MS signals with 100 ppm spiperone. As the concentration of the CHCA increases, the intensity of the analyte signal increases under the same analysis condition. There is an up limit of the concentration of the matrix in the solution for the matrix-embedded resorcinol formaldehyde solution to polymerize which is 20,000 ppm. When 40,000 ppm of the matrix is added, the polymer (RF-24) forms a lot of cracks which is not suitable as a MALDI-MS substrate. RF-16, which contained 20,000 ppm CHCA, produced satisfactory MS analysis result and is used throughout the rest of the MS analysis.

The solution is colorless while polymer polymerized from the solution is dark red. The color change indicates the formation of bonds on the conjugated structure which is the aromatic rings in this reaction. The mechanism of the resorcinol formaldehyde polymerization shown in Figure 2-5 is proposed by Lin and Ritter\textsuperscript{111} which has an addition and a condensation step. Base was used as the catalyst in the addition step. In the second step the acid was used as catalyst to cure the solution. In the experiment reported here only base, only acid, and base first then acid
as catalyst(s). In all cases there are successful examples, with the base catalyzed solution took longer time than the acid catalyst solution to polymerize. Figure 2-6 is the mechanism I proposed for the base-only catalyzed resorcinol formaldehyde polymerization reaction.

Analysis of the Spiperone on the CHCA Derived RF Polymer for Lower Background Interference from Matrix Molecules

This comparative analysis was performed with a microprobe quadrupole ion trap mass spectrometer (QITMS) which was built in the Yost laboratory by Christopher Reddick in 1997. The UV laser (Laser Science Inc. model VSL-337ND) used for all MALDI MS analysis has 20 Hz pulsed nitrogen laser with a wavelength of 337.1 nm and a 3 ns pulse width. The maximum energy output of the laser is >250 μJ/pulse with a peak power of 85 kW. Since the laser is near-diffraction limited, the beam can be focused to a diameter within a few times the laser’s wavelength; for the studies reported here, the laser spot size ranged from a diameter of 25 to 50 μm. The laser is focused into the mass spectrometer chamber by a single fused silica lens (Melles-Griot) with a focal length of 25.4 cm as illustrated in Figure 2-7. The laser is externally triggered after a 1 ms delay by a Wavetek model function generator so the ion source gate has fully opened before ionization. The position of the sample plate can be adjusted manually along x and y axes. Photosensitive paper is used to determine the position of the laser beam with respect to the sample plate to align the incident laser light. The software used to control the mass scan and data acquisition has been developed in our research group which is used to control auxiliary modulation frequency and amplitude to extend mass range. As shown in Figure 2-8, ions are produced during the MALDI process, and then bend 90° into the ion trap by using a DC quadrupole deflector. This is to reduce the possibility of neutral collisions occurring within the ion trap caused by neutral molecules. After being ejected by the QIT from the exit end, ions are detected using a conversion dynode and electron multiplier which is behind
the exit end electrode of the QIT. The data taken by this instrument was converted to excel file to generate the spectrum.

The analyte used here, spiperone, is an antidepressant. Figure 2-9 shows the fragmentation pathways of spiperone. Different matrices such as DHB, sinapinic acid, caffeic acid, and CHCA have been embedded into the RF polymer and the matrices-embedded polymers have been tested for the ability to ionize spiperone. MS analysis showed that the RF polymer with CHCA was the only combination that generated a spiperone MS signal (Table 2-1 to 2-4). This phenomenon can be explained by the result from the UV–vis spectra of the various matrix-embedded RF polymers (Figure 2-10). The discontinuity of spectrum at 350 nm is caused by the changing of light source from UV to visible. The CHCA had the highest absorption at 337 nm at the same concentration compare with other matrices; the absorbance of the other matrices may render them less effective MALDI matrices. Figure 2-11 shows the UV absorbance of CHCA in acetone (purple), the pure RF polymer (blue), and the RF polymer embedded with CHCA (orange) which were analyzed on Varian Cary-100 Conc UV-visible spectrometer from Varian (Palo Alto, CA). The CHCA-embedded RF polymer showed UV absorption at 337 nm which is not the case in the pure RF polymer. Since the solution of CHCA in acetone shows high absorbance around 337 nm range, it is obvious that the CHCA-embedded RF polymer obtained its high UV absorbance at 337 nm from embedding CHCA into it.

A polymerized RF 16 (20,000 ppm CHCA embedded RF polymer) pellet was attached onto the stainless steel MALDI microprobe with double-sided tape. A 0.2 μL volume of 100 ppm spiperone was deposited onto each polymer directly with pipette. After five minutes the sample is ready for MALDI-MS analysis.
A 0.2 μL volume of 2mM CHCA was also deposited onto the stainless steel microprobe with pipette; it was dry after 5 min past, then 0.2 μL of 100 ppm spiperone was deposited onto the matrix spot with pipette.

The results are shown in Figure 2-12 a and b, each showing the average of six spectra. The spectrum of 100 ppm spiperone with 20,000 ppm CHCA on the stainless steel showed [M+H]^+ (m/z 396) peak together with fragments from CHCA (m/z 123.2 and m/z 172.5) which pose interference to the interpretation of the spectrum while the spectrum of 100 ppm spiperone on 2% CHCA-embedded RF polymer showed high [M+H]^+ (m/z 396) peak and no noticeable fragment from CHCA. By embedding matrix molecules into RF polymer, the matrix molecules form covalent bonds with the polymer and are fixed into the polymer. Figure 2-13 is the illustration of one of the several possible bindings. Since the covalent bond between the polymer structure and the matrix molecules are much stronger than the hydrogen bond or the van der Waals force and thus requires much more energy to break the bond, the polymer keeps the matrix from being ablated while the matrix still has the ability to absorb laser energy to transfer to spiperone molecules and provide H^+ for ionization. Moreover, while using the same laser power for both analyses, the spectrum of spiperone on CHCA-embedded RF polymer showed lower intensities of daughter ions of spiperone (m/z 291.5, 238; m/z 165.5, 885) than the spectrum of spiperone with CHCA on stainless steel (m/z 291.5, 700; m/z 165.5, 3760). Comparing these two spectra, the spectrum obtained with spiperone on the CHCA-embedded RF polymer showed improved quality.

**Analysis of the Spiperone on the CHCA-Derived RF Polymer vs. Analysis of the Spiperone on the Stainless Steel Using CHCA as Matrix for More Uniform Analyte Signal Intensity Across the Sample**

The LTQ linear ion trap with vMALDI™ ion source from ThermoFinnigan (Figure 2-14) which is capable of imaging was used in this experiment. The laser power is 250 μJ/ pulse. The
laser beam was set up to move in 120 µm steps across the sample spot (3mm in diameter) and the data are collected automatically by the Xcalibur™ data system to generate the image of the signal intensity of the analyte throughout the analyzed area.

The dried-droplet method was chosen for the comparison experiment because this method is still widely used for MALDI-MS analysis despite the fact that the matrix-analyte co-crystals are unevenly distributed across the sample which causes uneven distribution of signal intensity of analyte across the sample spot. It would be exciting to see the improvement the CHCA-embedded RF polymer can make by simply using dry droplet method, and make the using dried-droplet method by mass spectrometry analyst with less effort needed for search of “sweet spots”.

For comparison of the dry droplet on stainless steel analysis vs. spiperone solution on CHCA embedded RF polymer analysis; 0.2 µL of 0.5 M CHCA in MeOH was applied on the stainless steel; after the matrix solution was dried, 0.2 µL of 100 ppm spiperone solution in MeOH was applied on the dried matrix spot and on the CHCA embedded RF polymer, respectively. The microscopic image of the sample spot prepared with dried-droplet method before MS analysis is shown in Figure 2-15. After the sample plate was inserted into the instrument, the image of the sample plate was taken by the CCD camera and a circle was drawn by hand. The instrument started to run MALDI analysis throughout the circled area with each analysis spot 120 µm apart both in x direct and y direction. The position parameters (x- and y-axis) were recorded as attachment of each spectrum for each analysis spot by the vMALDI software to be used to generate the image of the sample spots with the information of mass spectra after the analysis. Both image of the distribution of intensity of TIC (total ion counts) and image of the distribution of intensity of the ions within certain range can be generated. RSD, relative standard deviation which is calculated by dividing the standard deviation by the average
value, was calculated for \( m/z \) 304 (for cocaine) by surfer 8 based on all the spectra taken throughout the circled area and reported in “grid report”. Typical results from the sample prepared with dry droplet method on the stainless steel sample plate are shown in Figure 2-16 with relative standard deviation (RSD) as 228%.

Figure 2-17 is the microscopic image of the CHCA-embedded RF polymer pellet after the deposition of 0.2 μL of 100 ppm spiperone. Same mass spectrometry analysis as for the dried-droplet of spiperone on stainless steel was run for dried-droplet of spiperone on CHCA-embedded RF polymer. Both image of the distribution of intensity of TIC (total ion counts) and image of the distribution of intensity of the ions within certain range can be generated. RSD was calculated for \( m/z \) 304 (for cocaine) by surfer 8 based on all the spectra taken throughout the circled area and reported in “grid report”. Typical results from the sample prepared with dry droplet method on the CHCA-embedded RF polymer are shown in Figure 2-18 with relative standard deviation (RSD) as 103%.

Compared to the results obtained from the dry droplet method on the stainless steel (Figure 2-16), the results from the dried-droplet on the CHCA-embedded RF polymer (Figure 2-17) produced more even distribution of the analyte across the sample with the RSD of the sample prepared on the CHCA-embedded RF polymer is about the half of that with the sample prepared with the dried-droplet method on stainless steel; thus, analyte signals can be detected easily without searching for the “sweet spot”. The more even distribution of the analyte signal resulted from the incorporation of matrix molecules into the rigid polymeric structure of RF polymer, since the matrix molecules are evenly distributed throughout the whole polymeric structure on the molecular level. The morphology of the analyte crystals only depends on the distribution of the analyte across the sample and no concern of distribution of matrix molecules is necessary.
Conclusion

With the 1, 4 polymerization position on the aromatic ring, RF polymers can withstand the harsh condition of chemical modification and washing with aqueous solution which is the condition for most biological sample. With the aromatic groups in the RF polymers and reaction sites on the aromatic rings it is possible to incorporate aromatic groups containing matrix molecules in the polymeric network with covalent bonds. By trapping the matrix molecules in the polymeric network with covalent bonds it still provides the assistant to ionization but can reduce the amount of matrix molecules been ablated by laser which can interfere with the MS signals from analyte with low molecular weight because it needs more energy to break a covalent bond than to break a intermolecular noncovalent bond. By incorporating the matrix molecules into the polymeric network structure, it can help solve the problem of unevenly distributed signal throughout the sample plate which is always a problem in MALDI-MS analysis. Many different sample preparation methods were developed to minimize the problem. The new resorcinol-formaldehyde (RF) polymers developed have physical and chemical properties suitable for embedding matrix molecules in the substrate for lowering the background noise from matrix molecules. With the nature of the matrix-embedded polymer the matrix molecules distributed in the polymer evenly at the molecular level, thus provide the possibility to produce uniform signal intensity of analyte across the sample. This property also provides the possibility for quantization of the analyte in MALDI which is an important issue related to MALDI sample preparation.
Figure 2-1. The mechanism of the TEOS sol-gel reaction. [Adopted from Lin, Y.; Chen, Y. Laser desorption/ionization time-of-flight mass spectrometry on sol-gel-derived 2,5-dihydroxybenzoic acid film Analytical Chemistry 2002, 74, 5793]
Figure 2-2. Illustration of incorporation of DHB molecules in the TEOS gel. [Adopted from Lin, Y.; Chen, Y. Laser desorption/ionization time-of-flight mass spectrometry on sol-gel-derived 2,5-dihydroxybenzoic acid film *Analytical Chemistry* 2002, 74, 5793]
Figure 2-3. Microscope pictures showing the sample surfaces of manually prepared samples with the dried-droplet (DD) or the seed layer (SL) method using (a) α-cyano-hydroxycinnamic acid (CHCA) DD, (b) CHCA SL, (c) sinapinic acid (SA) DD, (d) SA SL, (e) ferulic acid (FA) DD, (f) FA SL. [Adopted from Onnerfjord, P.; Ekstrom, S.; Bergquist, J.; Nilsson, J.; Laurell, T.; Marko-Varga, G. Homogeneous sample preparation for automated high throughput analysis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry \textit{Rapid Communications in Mass Spectrometry} \textbf{1999}, \textit{13}, 315]
Figure 2-4. MALDI ion images showing intensities for selected peptides using (A) the thin-film, α-CHC sample and (B) the dried-droplet, DHB sample. Approximately 250 fmol of α₁-₈, 250 fmol of AP, and 1 pmol of ELH were prepared with each matrix. The black-to-red color map corresponds to the arbitrary intensity values specified for each peptide, while the grid lines correspond to 50 μm increments within each image. [Adopted from Garden, R.W.; Sweedler, J.V. Heterogeneity within MALDI samples as revealed by mass spectrometric imaging Analytical Chemistry 2000, 72, 30]
1. Addition Reaction

2. Condensation Reaction

Figure 2-5. Mechanism of the polymerization of RF polymer proposed by Lin and Ritter. [Adopted from AL-Muhtaseb, S.A.; Ritter, J.A. Preparation and properties of resorcinol-formaldehyde organic and carbon gels *Advanced Materials* **2003**, *15*, 101]
Figure 2-6. Base-catalyzed RF polymerization.
Figure 2-7. Cross-section of the ion trap mass spectrometer. [Adopted from Reddick, C.D. The detection of pharmaceutical drug compounds from intact biological tissue by matrix-assisted laser desorption ionization method (MALDI) quadrupole ion trap mass spectrometry 1997 PhD dissertation. University of Florida, Gainesville, Florida.]
Figure 2-8. Illustration of the path of laser and ions in mass spectrometer. [Adopted from Reddick, C.D. The detection of pharmaceutical drug compounds from intact biological tissue by matrix-assisted laser desorption ionization method (MALDI) quadrupole ion trap mass spectrometry 1997 PhD dissertation. University of Florida, Gainesville, Florida.]
Figure 2-9. Fragment path ways of spiperone.
Figure 2-10. UV absorbances of the various matrix-embedded RF polymers.
Figure 2-11. UV absorbances of the RF polymer (blue), CHCA-embedded RF polymer (orange), and CHCA solution (pink). The spike at 320 and 350 is caused by the instrument used for analysis.
Figure 2-12. Mass spectrum of the 100 ppm spiperone on A) 20,000 ppm CHCA embedded RF polymer, and B) stainless steel with CHCA as matrix.
Figure 2-13. CHCA embedded in the RF polymer structure.
Figure 2-14. LTQ with vMALDI ion source from ThermoFinnigan.
Figure 2-15. The microscopic image of the sample well after the dry droplet sample preparation before MS analysis (3 mm in diameter).
Figure 2-16. The distribution of the spiperone intensity ($m/z$ 396) across the sample using dry droplet method on stainless steel. A) 2-D image. B) 3-D image.
Figure 2-17. The microscopic image of the RF-16 polymer pellet after the deposition of 0.3 μL of 100 ppm spiperone before MS analysis.
Figure 2-18. The distribution of the spiperone intensity (m/z 396) across the sample using dry droplet method on RF-16 polymer pellet. A) 2-D image. B) 3-D image.
<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Matrix</th>
<th>Polymerized</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF-1</td>
<td>0.0078 g Na₂CO₃ (R/C 1000) no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>RF-2</td>
<td>0.0156 g Na₂CO₃ (R/C 500) no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>RF-39</td>
<td>0.0156 g Na₂CO₃ (R/C 100) no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>RF-3</td>
<td>0.0156 g Na₂CO₃ (R/C 500) 0.0168 g DHB (1000 ppm) yes</td>
<td>no</td>
<td>N/A</td>
</tr>
<tr>
<td>RF-20</td>
<td>0.039 g Na₂CO₃ (R/C 100) 0.672 DHB (40,000 ppm) yes</td>
<td>no</td>
<td>N/A</td>
</tr>
<tr>
<td>RF-27</td>
<td>0.0156 g Na₂CO₃ (R/C 500) 0.4146 g CHCA (20,000 ppm) + 0.3492 g 4-(imidazole-1-yl)phenole (20,000 ppm) no</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>RF-28</td>
<td>0.0156 g Na₂CO₃ (R/C 500) 0.4146 g CHCA (20,000 ppm) + 0.3492 g 4-(imidazole-1-yl)phenole (20,000 ppm) 2 h later add 1.6 mL 0.1 M HCl to cue no</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>RF-29</td>
<td>0.0156 g Na₂CO₃ (R/C 500) 0.2073 g CHCA (10,000 ppm) + 0.2746 g 4-(imidazole-1-yl)phenole (16,000 ppm) 2 h later add 1.6 mL 0.1 M HCl to cue yes</td>
<td>no</td>
<td>N/A</td>
</tr>
<tr>
<td>RF-30</td>
<td>0.156 g Na₂CO₃ (R/C 50) 0.2073 g CHCA (10,000 ppm) + 0.2746 g 4-(imidazole-1-yl)phenole (16,000 ppm) yes</td>
<td>no</td>
<td>N/A</td>
</tr>
<tr>
<td>RF-31</td>
<td>0.156 g Na₂CO₃ (R/C 50) 0.2073 g CHCA (10,000 ppm) + 0.2746 g 4-(imidazole-1-yl)phenole (16,000 ppm) 2 h later add 1.6 mL 0.1 M HCl to cue yes</td>
<td>no</td>
<td>N/A</td>
</tr>
<tr>
<td>RF-32</td>
<td>0.078 g Na₂CO₃ (R/C 100) 0.2073 g CHCA (10,000 ppm) + 0.2746 g 4-(imidazole-1-yl)phenole (16,000 ppm) no</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>RF-33</td>
<td>0.078 g Na₂CO₃ (R/C 100) 0.2073 g CHCA (10,000 ppm) + 0.2746 g 4-(imidazole-1-yl)phenole (16,000 ppm) 2 h later add 1.6 mL 0.1 M HCl to cue no</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>RF-34</td>
<td>0.034 g Na₂CO₃ (R/C 200) 0.2073 g CHCA (10,000 ppm) + 0.2746 g 4-(imidazole-1-yl)phenole (16,000 ppm) no</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>RF-35</td>
<td>0.034 g Na₂CO₃ (R/C 200) 0.2073 g CHCA (10,000 ppm) + 0.2746 g 4-(imidazole-1-yl)phenole (16,000 ppm) 2 h later add 1.6 mL 0.1 M HCl to cue no</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Catalyst</td>
<td>Matrix</td>
<td>Polymerized</td>
<td>Signal</td>
</tr>
<tr>
<td>----------</td>
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<td>-------------</td>
<td>--------</td>
</tr>
<tr>
<td>RF-4</td>
<td>0.0078 g Na2CO3 (R/C 1000)</td>
<td>0.0168 g DHB (1000 ppm)</td>
<td>Crystalline</td>
</tr>
<tr>
<td>RF-5</td>
<td>0.0156 g Na2CO3 (R/C 500)</td>
<td>0.0168 g DHB (1000 ppm)</td>
<td>Crystalline</td>
</tr>
</tbody>
</table>
Table 2-3. RF polymers with water as solvent and Na$_2$CO$_3$, or HCl, or HClO$_4$ as catalyst: 4.00 g Resorcinol + 5.00 mL formaldehyde in 12.00 mL H$_2$O

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Matrix</th>
<th>Polymerized</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF-19</td>
<td>0.0156 g Na$_2$CO$_3$ (R/C 50)</td>
<td>no</td>
<td>too quick</td>
</tr>
<tr>
<td>RF-6</td>
<td>0.0156 g Na$_2$CO$_3$ (R/C 500)</td>
<td>0.0168 g DHB (1000 ppm)</td>
<td>yes (5 hrs)</td>
</tr>
<tr>
<td>RF-7</td>
<td>0.0156 g Na$_2$CO$_3$ (R/C 500)</td>
<td>0.0168 g DHB (1000 ppm) + 0.266 g FeCl$_3$·6H$_2$O (100 ppm)</td>
<td>too quick (15 mins)</td>
</tr>
<tr>
<td>RF-8</td>
<td>0.0156 g Na$_2$CO$_3$ (R/C 500)</td>
<td>0.0168 g DHB (1000 ppm) + 0.0266 g FeCl$_3$·6H$_2$O (100 ppm)</td>
<td>yes (2 hrs)</td>
</tr>
<tr>
<td>RF-9</td>
<td>0.0156 g Na$_2$CO$_3$ (R/C 500)</td>
<td>0.0336 g DHB (2000 ppm) + 0.0266 g FeCl$_3$·6H$_2$O (100 ppm)</td>
<td>yes (3 hrs)</td>
</tr>
<tr>
<td>RF-10</td>
<td>0.0156 g Na$_2$CO$_3$ (R/C 500)</td>
<td>0.0336 g DHB (2000 ppm)</td>
<td>yes (2 hrs)</td>
</tr>
<tr>
<td>RF-11</td>
<td>0.0156 g Na$_2$CO$_3$ (R/C 500)</td>
<td>0.168 g DHB (10,000 ppm)</td>
<td>yes (2 hrs)</td>
</tr>
<tr>
<td>RF-12</td>
<td>0.1737 mL 1 M HClO$_4$ (R/C 200)</td>
<td>0.168 g DHB (10,000 ppm)</td>
<td>yes (1 hr)</td>
</tr>
<tr>
<td>RF-15</td>
<td>1.6 mL 0.1 M HCl (R/C 200)</td>
<td>0.336 g DHB (20,000 ppm)</td>
<td>yes (&lt; 0.5 hr)</td>
</tr>
<tr>
<td>Catalyst</td>
<td>Matrix</td>
<td>Polymerized</td>
<td>Signal</td>
</tr>
<tr>
<td>----------</td>
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<td>--------</td>
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<tr>
<td>RF-38</td>
<td>1.6 mL of 0.1 M HCl (R/C 200) no</td>
<td>yes</td>
<td>no</td>
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<tr>
<td>RF-17</td>
<td>1.6 mL of 0.1 M HCl (R/C 200) 0.49 mL 1-vinylimidazole (100,000 ppm)</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>RF-18</td>
<td>1.6 mL of 0.1 M HCl (R/C 200) 0.1593 g EDTA (10,000 ppm)</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>RF-26</td>
<td>1.6 mL of 0.1 M HCl (R/C 200) 0.3492 g 4-(imidazole-1-yl)phenole (20,000 ppm) yes (&lt; 0.5 hr) sol became milky in freezer</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>RF-14</td>
<td>1.6 mL of 0.1 M HCl (R/C 200) 0.336 g DHB (20,000 ppm)</td>
<td>yes (1 hr) yes</td>
<td></td>
</tr>
<tr>
<td>RF-16</td>
<td>1.6 mL of 0.1 M HCl (R/C 200) 0.4146 g CHCA (20,000 ppm)</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>RF-24</td>
<td>1.6 mL of 0.1 M HCl (R/C 200) 0.8292 g CHCA (40,000 ppm)</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>RF-25</td>
<td>1.6 mL of 0.1 M HCl (R/C 200) 0.2073 g CHCA (10,000 ppm)</td>
<td>yes yes</td>
<td></td>
</tr>
<tr>
<td>RF-21</td>
<td>1.6 mL of 0.1 M HCl (R/C 200) 0.488 g SA (20,000 ppm)</td>
<td>yes no</td>
<td></td>
</tr>
<tr>
<td>RF-22</td>
<td>1.6 mL of 0.1 M HCl (R/C 200) 0.390 g caffeic acid (20,000 ppm)</td>
<td>yes no</td>
<td></td>
</tr>
<tr>
<td>RF-23</td>
<td>1.6 mL of 0.1 M HCl (R/C 200) 0.312 g 3-amino-qumiline (20,000 ppm)</td>
<td>yes no</td>
<td></td>
</tr>
<tr>
<td>RF-36</td>
<td>3.2 mL of 0.1 M HCl (R/C 100) 0.2073 g CHCA (10,000 ppm) + 0.2746 g 4-(imidazole-1-yl)phenole (16,000 ppm) no</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>RF-37</td>
<td>0.16 mL of 1 M HCl (R/C 200) 0.2073 g CHCA (10,000 ppm) + 0.2746 g 4-(imidazole-1-yl)phenole (16,000 ppm) no</td>
<td>no</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3
SURFACE MODIFIED RF POLYMER WITH APTAMERS: METHODOLOGY AND USAGE
IN SELDI-MS SAMPLE PREPARATION

Because of the complexity of biological samples obtained from individuals for disease screening tests and the chemicals used during the sample handling process, one or more cleaning or separation steps is usually employed to remove most of the unwanted species and pre-concentrate the biomarkers before MALDI-MS analyses. Those separation and enrichment methods are usually tedious, time-consuming, and require large amounts of sample.

Many investigations have been performed to reduce the interferences from the uninformative components in MALDI analyses without a cleaning step. Several polymers were used to achieve on-probe reduction of contaminations. High-density polyethylene membrane, high-density polypropylene, octyl (C$_8$) and octadecyl (C$_{18}$) extraction disk from 3M (St. Paul, MN) were used successfully to remove salt, detergent, and glycerol contaminants from peptide and protein sample solutions. Immobilization of proteins onto nylon-66 and positive charge-modified nylon (Zetabind) membranes removed the water soluble contaminants before MS analysis. Perfluorosulfonated ionomer film were used to analyze biological mixtures such as chemical digests of proteins, proteins in milk and egg white, cell lysate, and oligonucleotide without pre-purification with enhanced results. On-probe purification also achieved by using digests nitrocellulose for MALDI-MS analysis of PCR (Polymerase chain reaction) products and DNA fragment. Polyethylene membrane was used for high-mass proteins (100,000 Da) MALDI-MS analysis with enhanced spectral quality. Hydrophilic poly(acrylic acid) (PAA) was used for its selective adsorption of partially digested myoglobin to remove contaminants by rinsing with water. Metals such as patterned gold with poly(acrylic acid) as analysis surfaces were tested to reduce the contaminations in the samples.
Surface modifications have been performed in several cases with polymeric materials in order to covalently bind a certain class of analyte for purification from solution for enhanced performance (SELDI). A surface of poly(4-vinylpyridine) which was extended with spacer arms (-N-ethylsuccinamyl-) and terminated with leaving groups (N-hydroxysulfosuccinimide) for covalent attachment of an 18-residue peptide (N terminus of human ß-casein) was used for on-probe purification for pre-analysis purification. Poly-lysine-immobilized poly-2-hydroxyethyl methacrylate membrane was used to selectively absorb DNA from aqueous solution.

Affinity adsorption without the formation of covalent bonds allows relatively easy mass spectrometric analysis. Surface modification, which adds specific affinity to the substrate surface for affinity extraction, was developed. The extremely strong avidin glycoprotein-biotin interaction ($k_d = 10^{-15}$) was utilized to retain the biotinylated analyte onto the avidin immobilized agarose beads or polymer thin films. Lectins have been immobilized onto gold foils to affinity capture bacteria from the contents in the sample solution. Several dextrans were immobilized onto the fresh gold support as a self-assembled monolayer to retain binding molecules which capture analytes of interest. DNA has been immobilized onto the poly(ethylene terephthalate) microfiber to capture anti-DNA antibodies.

Although surface modification with affinity agents is superior to simply using the polymers as substrates for MALDI-MS analysis, each of the methods has its limitation. The avidin modified substrate requires the analyte to be biotinylated; lectins can be used to capture bacteria but lacks specificity; the dextran-modified gold surface can work the same way as IMAC does and both lack specificity; DNA has higher specificity but with the drawback of difficulty in obtaining and handling DNA, plus the large size of DNA causes less amount to be attached onto the solid support. A universal one-step on-probe clean-up method which has a
high specificity towards biomarkers, high enough density of recognizing sites, with low cost and yet easy to manipulate for biological sample analysis has been developed. This method is a surface modification of a RF polymer with aptamers for affinity capture. Antibodies have the highest specific affinity among the biological receptors used for modifications, though is one of the most expensive. Aptamers have numerous advantages over antibodies, yet, have not been wildly tested.

As shown in Figure 3-1, there are two phenol groups on each aromatic ring which make up most of the RF polymer structure. These phenol groups provide plenty of reaction sites for direct surface modification with aptamer and surface modification with aptamer via carboxylation, i.e., indirect surface modification. The phenol groups on the RF polymer have a pKa of 9.86 and the hydroxyl groups on the silica particle surface have a pKa of 9.82; thus, the reactivity of the phenol groups should be very close to the hydroxyl groups on the glass nanoparticles. The surface modification of the RF polymer can follow the procedures used in the research lab of Dr. Weihong Tan (University of Florida) for the surface modification of silica particles.

**Aptamers Used for RF Polymer Surface Modification**

Aptamers have been used as molecular beacons in Weihong Tan’s lab (University of Florida) for in vitro fluorescent detection. Molecular beacons detect the existence of an analyte according to the changing fluorescent signal intensity when the beacon changes the conformation as the result of the affinity capture of the analyte. Fluorescent signal detection for analyte with molecular beacon works very well if the analyte of detection in known to exist in the tested solution, otherwise, there can be a false positive result and a definite identification is impossible. Tandem mass spectrometry analysis (MS^n) has the capability for definite identification of a substance based on its fragmentation pattern. Coupling the high specific recognition capability of molecular beacon with the informing power of mass spectrometry would be the answer for the
limitations of molecular beacons. Thrombin-binding aptamers were covalently attached to the surface of a glass slide and were used successfully for affinity capture of thrombin in MALDI targets by McGown at Duke University. The RF polymer developed in our lab has enough reaction sites, with similar reactivity as silica slides, to be used as solid support for aptamer attachment and with the flexibility in the size and shape the sol-gel polymerization method renders.

There is a long list of aptamers has been generated by SELEX process; moreover, an aptamer can be produced with affinity towards any specific target molecule by the SELEX process. The choice of the aptamer to be attached onto the RF polymer is based on the concern that the successful attachment of the aptamers onto the RF polymer should be verified before the affinity capture of a specific molecule is proved by mass spectrometric analysis. Verification of the attachment of the aptamers on the RF with MS would include MS-related variables into the verification process; thus, include more uncertainty in the detection. Since the task in this project is to develop a methodology for affinity capture of analyte of interest prior to MS analysis, choosing an existing aptamer with high affinity of a specific molecule would save a lot of effort in generating a new aptamer. An aptamer with high affinity towards a molecule that can be detected by the naked eye and be measured by UV–vis was chosen.

The molecule chosen is reactive green 19 and belongs to a group of reactive dyes used in textile industry. This group of dyes has been used in dye-ligand affinity chromatography for the purification of proteins because of the high selectivity and the reversibility of the affinity binding between the dye and the protein. These synthetic dyes interact with the active sites of many proteins by mimicking the structure of the substrates, cofactors, or binding agents for these proteins. The aromatic triazine dye structure resembles the nucleotide structure of nicotinamide
adenine dinucleotide and the dye interacts with the dinucleotide fold in these proteins. These reactive dyes can bind proteins by electrostatic and hydrophobic interactions and by more specific “pseudoaffinity” interactions with ligand-binding sites. The degree of purification achieved with dye-ligand chromatography is generally better than that obtained with less specific techniques such as ion-exchange or gel filtration chromatography. The reactive dyes are relatively inert and unaffected by enzymes in crude cellular extracts. Figure 3-2 shows the structure of the reactive green 19 molecule. This dye molecule has absorbance peak at around 630 nm wavelength and can be detected by the naked eye and UV–vis spectrometry.

The sequence of the aptamer with high specific affinity toward reactive green 19 is ACCCG GCGTT CGGGG GGTAC CGGGT which was discovered by Ellington and Szostak in 1992 and can be synthesized in a DNA synthesizer. In 20 mM Tris buffer at pH 7.6 with 0.5 M LiCl and 1 mM MgCl2, the aptamer folds into a stem-loop structure as shown in Figure 3-3 and can be used in a bioassay when labeled with fluorescein.

After the reactive green 19-retaining aptamer was successfully attached onto the RF polymer, a cocaine-retaining aptamer was chosen to be attached onto the RF polymer for the cocaine capturing and MS analysis. Cocaine is an alkaloid found in leaves of the South American shrub Erythroxylon coca. The active ingredient of the coca plant was first isolated in the West by the German chemist Friedrich Gaedcke in 1855. Albert Niemann described an improved purification process for his PhD which he named as "cocaine". Cocaine is a powerfully addictive stimulant drug which was tried by approximately 33.7 million Americans ages 12 and older, representing 13.8% of the population, at least once in their lifetimes according to the 2005 National Survey on Drug Use and Health. Although detection of cocaine by GC-MS is well established, it will be helpful if cocaine can be captured from biological sample such
as blood and urine and to be analyzed by MALDI-MS. Actually, after this aptamer affinity capture procedure is established, any disease biomarker could be captured and analyzed by MS the same way as reactive green 19 and cocaine.

Aptamer affinity capture of cocaine can be achieved by attaching a cocaine-retaining aptamer onto the RF polymer. An aptamer with the affinity towards cocaine (Figure 3-4 b), one of the two cocaine isomers, was constructed by Landry et al., as shown in Figure 3-5 which forms a three-way junction in 20 mM Tris buffer with pH 7.4, 140 mM NaCl, and 2 mM MgCl₂ to accommodate a cocaine molecule at the center of the aptamer tertiary structure. With dissociation constant of $0.4 \text{ – } 10 \mu\text{M}$ between cocaine and the cocaine-retaining aptamer⁴⁵, cocaine-retaining aptamer was used as a fluorescent sensor for cocaine⁴⁵ and colorimetric probe for the detection of cocaine.¹³³

**Direct Surface Modification of the RF Polymer with Aptamer**

The reactive green 19-retaining aptamer and cocaine-retaining aptamer were directly attached onto the RF polymer in the nucleic acid synthesizer (Applied Biosystems 3400 DNA Synthesizer, Figure 3-6). The Applied Biosystems 3400 DNA Synthesizer from Applied Biosystems (Foster City, CA) is a fully programmable instrument that provides four-column simultaneous synthesis, and features automatic base dilution and analysis of coupling efficiency.

The RF polymer pellets were first polymerized by depositing RF solution onto the top of a stainless steel microprobe and allowing several hours for the solution to polymerize. These pellets were then hammered to small pieces to fit in the columns of the DNA synthesizer. These RF polymer pieces were loaded into the reaction column as solid support for aptamer attachment. The sequence of the aptamer was set up using the software and the synthesis of the aptamers was controlled by the computer. In each step, the solution is pumped through the column which is attached to the reagent delivery lines and the nucleic acid synthesizer. Each base is added via
computer control of the reagent delivery. After the synthesis was finished the RF polymer pieces were taken out to be immersed in \( \text{NH}_2\text{OH} / \text{CH}_3\text{NH}_2 \) ratio as one at room temperature for three hours for de-protection of the aptamers.

**Surface Modification of the RF Polymer with Aptamers via Surface Modification of the RF Polymer with Carboxylic Groups**

Research has been conducted in the research group of Dr. Weihong Tan group on carboxylation of silica nanoparticles and reaction of the carboxylic acid groups with amine modified aptamers for indirect surface modification. According to the procedure, the RF polymer will be carried out by immersion in freshly prepared 1\% (v/v) solution of distilled (3-(trimethoxysilyl)propyl)diethylenetriamine (DETA) and 1 \text{mM} acetic acid for 30 min at room temperature. The DETA modified RF polymer will be rinsed with deionized water to thoroughly remove excess DETA. The amine-functionalized polymer was then treated with succinic anhydride in dry tetrahydrofuran (THF) in presence of an argon atmosphere for 6 h. Figure 3-7 shows the scheme of the surface modification.

After the RF polymer was carboxylated amine-modified cocaine aptamers were attached onto the RF polymer via the formation of an amide bond following the procedure used by Weihong Tan’s lab.\(^{136}\)

**Characterization of the Carboxylic Group-Modified RF Polymer**

The verification of the carboxylation was performed by labeling the RF polymer with 1-pyrenyl diazomethane (PDAM) (Figure 3-8), a new fluorescent labeling reagent from Molecular Probes (Eugene, OR) with an amine group on it.\(^{139}\) The covalent attachment of the PDAM to the carboxylated RF polymer was performed according to the procedure described by Gaber et al.\(^{139}\) The carboxylated RF polymer pieces (sample) and non-carboxylated RF polymer pieces (control) were placed in two separate vials and immersed in an aliquot of 10 \text{mM} PDAM solution
in ethanol. The two suspensions were mixed vigorously on a vortex mixer for two hours at room temperature before the polymer pieces were thoroughly rinsed with ethanol. The polymer pieces were transferred to two separate vials and were rinsed three times with ethanol.

The excitation wavelength of PDAM is 351 nm and the fluorescent emission wavelength is 392 nm. Fluorescence emission intensity spectra of the sample and control in ethanol were obtained by readings from Tecan Microplate Reader from Tecan (Mannedorf/Zurich, Switzerland) to verify the success of surface modification of the RF polymer (Figure 3-9). The PDAM-bond polymer pieces has a much higher emission at 392 nm wavelength after excited at 351 nm than the control which proved the existence of carboxylic acid groups on the RF polymer.

The determination of the amount of the carboxylic groups on the RF polymer was evaluated from the uptake of a basic dye molecule, toluidine blue O (Figure 3-10)\textsuperscript{140} by the carboxylated RF polymer. Toluidine blue O complexes to equivalent moles of carboxylate groups which is the base for calculating the amount of the carboxylic acid groups according to the amount of the toluidine blue O absorbed by the carboxylated RF polymer.\textsuperscript{140} The carboxylated RF polymer pieces were immersed in 500 μm toluidine blue O of pH 10 at 30 °C for five hours before the RF polymer pieces were rinsed thoroughly with 5.0 x 10\textsuperscript{-4} N NaOH aqueous solution. The polymer pieces were rinsed three times after they were transferred to another vial. The dye molecules were desorbed from the RF polymer by immersing the RF polymer pieces in 50% acetic acid solution.

All the UV spectra in this project were recorded on Varian Cary-100 Conc UV-visible spectrometer (Palo Alto, CA). Toluidine blue O has UV absorbance at 285 and 623 nm. The UV absorbance of the toluidine blue O desorbed from the carboxylated and noncarboxylated RF
polymer served as a second proof of the existence of the carboxylic acid groups on the RF polymer (Figure 3-11).

The UV absorbance of the toluidine blue O solutions with different concentrations (Figure 3-12) was used to construct a calibration curve at 285 nm (Figure 3-13). Bases on the linear regression fit shown, the concentration of the toluidine blue O desorbed from twelve round pieces (3.5 mm diameter) of carboxylated RF polymer and non-carboxylated RF polymer in 9 mL of the 50% acetic acid solution was 7.8x10⁻⁶ M and 4.8x 10⁻⁶ M, respectively. The difference of the concentration of toluidine blue O desorbed between the carboxylated RF and non-carboxylated RF polymer pieces was 3.0x10⁻⁶ M mol by subtracting the concentration of toluidine blue O desorbed from the noncarboxylated RF polymer from the concentration of the toluidine blue O desorbed from the carboxylated RF polymer. The difference of the amount of toluidine blue O in 9 mL of solutions between the carboxylated RF and non-carboxylated RF polymer pieces was calculated by timing the difference of the concentration of toluidine blue O between the carboxylated RF and non-carboxylated RF polymer pieces with 9 mL to get 2.7x10⁻⁸ M. The density of the carboxylic groups on the polymer was calculated by dividing 2.7x10⁻⁸ mol by the total surface area of the twelve polymer pieces with two sides (231 mm²) to get 1.2x10⁻¹⁰ mol/mm². The number of carboxylic groups available to be ablated by a 100 micrometer radius laser pulse is thus 3.7x 10⁻¹² mol, which is more than adequate to yield a strong MALDI signal.

**Affinity Capture of RG 19 with RG 19-Retaining Aptamer-Modified RF Polymer and Characterization of the Modified RF Polymer**

The affinity capture of the RG 19 by the RG 19-retaining aptamer was demonstrated by desorption of this dye as detected by UV–vis spectrometry. The aptamer-modified RF polymer pieces and non-modified RF polymer pieces were immersed in the 20 mM Tris-Cl buffer with
RG 19 in two separate plastic vials for four hours. The polymer pieces were rinsed thoroughly with the Tris buffer before they were transferred to two different vials in order to avoid the non-specific capture of the RG 19 on the plastic vial wall. The polymers were rinsed three times again before deionized (DI) water was added to the vial to desorb the RG 19. The DI water in the vial containing the aptamer-attached RF polymer turned blue instantly, while the DI water in the vial contained the non-modified RF polymer did not change color. UV spectrum was taken for the desorption solution from both vials (Figure 3-14).

In order to determine the amount of aptamer on the polymer surface, the concentration of RG 19 desorbed into solution was measured by its UV absorbance. A calibration curve was constructed (Figure 3-15) according to the UV absorbance at 634 nm of the reactive green 19 solutions different concentration. The equation of the calibration curve as \( y=0.00061x + 0.0019 \). According to the calibration curve the amount of the RG 19 was calculated to be 0.20 mM from the 0.123 UV absorbance of the sample. The amount of the RG 19 in the 5 mL solution was 7.8x10^{-7} mol. Two round pieces of the RF polymer have a total surface area of 38.5 mm². The density of the reactive green 19 absorbed on the aptamer-attached polymer was calculated as 2.03x10^{-8} mol/mm². The number of reactive green 19 can be ablated by laser (the radius of the laser shot is estimated to be 100 μm) is 6.0x10^{-10} mol /spot, which is plenty for MALDI-MS analysis.

**Affinity Capture of Cocaine with Direct and Indirect Cocaine–Retaining Aptamer-Modified RF Polymer**

The affinity capture of cocaine from a cocaine solution by the direct aptamer modified RF polymer was first detected using a visual detection method, conducted according to the procedure described by Landry et al. Diethylthiatricarbocyanine iodide, a cyanine dye, can be captured by the cocaine-retaining aptamer. The addition of the cocaine solution into the
diethylthiatricarbocyanine iodide-aptamer complex containing would release the dye molecule to capture the cocaine molecule (Figure 3-16)\textsuperscript{53} which serves as an easy visual detection of the existence of the cocaine-retaining aptamers on the RF polymer. The pieces of the cocaine retaining-aptamer modified RF polymer and pieces of non-modified RF polymer were put into two separate vials with 500 μL of 20 mM Tris buffer. A 50 μL of 7 μM of diethylthiatricarbocyanine iodide in methanol was added into the vial followed by vigorous mixing on the Vortex for several seconds. After five minutes, both suspensions were thoroughly rinsed with buffer solution and transferred to two vials. The polymer pieces were rinsed with buffer three times before 5 μL of cocaine solution (1 mg/mL acetonitrile) was added into both vials with 500 μL Tris buffer. The solution in the vial with non-modified RF polymer pieces did not change color, while the solution in the vial with the cocaine retaining-aptamer modified RF polymer changed from colorless to blue. The color of the solution faded several hours later due to the hydrolysis of the dye molecules in the slightly basic buffer.\textsuperscript{51}

MALDI-MS spectra of the cocaine-retaining-aptamer modified RF polymer and unmodified RF polymer were taken on the LTQ with vMALDI source. Three pieces of cocaine retaining-aptamer modified RF polymer and three pieces of non-modified RF polymer were immersed in 500 μL of 20 mM Tris buffer for the aptamers to form the affinity capturing configuration. Then 5 μL of cocaine solution (1 mg/mL acetonitrile, 3mM) was added into the vials, waited for five minutes before they were thoroughly rinsed with 20 mM Tris buffer solution. The polymer pieces were transferred to another two vials to be rinsed three times before they were taken out and inspected under the microscope (Figure 3-17).

The polymers were taped onto the sample slide with double-sided tape and inserted into the instrument. Sinapinic acid (SA), 2,5-dihydroxybenzoic acid (DHB), and α-cyano-4-
hydroxycinnamic acid (CHCA) were used as MALDI matrices on three of the modified polymer respectively and on three non-modified respectively. The cocaine retaining-aptamer modified RF polymer showed cocaine signal (the [M+H]$^+$ ion at $m/z$ 304) when SA was used as the matrix, meanwhile there was no cocaine signal when DHB or CHCA was used as matrices, which is consistent with the results McGown observed with SA for her aptamer-captured thrombin sample. The polymer without the cocaine retaining aptamer (Figure 3-18a) showed no cocaine ($m/z$ 304) compared to the cocaine retaining aptamer attached polymer (Figure 3-18b). The 2-D and 3-D images of the intensity of the $m/z$ at 304 ions of cocaine on the RF polymer is shown in Figure 3-19.

An indirect cocaine retaining-aptamer modification of the RF polymer was also conducted for the in Weihong Tan’s lab after the polymer pellets were carboxylated in our lab. Three pieces of cocaine retaining-aptamer modified RF polymer were immersed in 500 μL of 20 mM Tris buffer for the aptamers to form the affinity capturing configuration. Then 5 μL of cocaine solution (1 mg/mL acetonitrile, 3mM) was added into the vial, waited for five minutes before they were thoroughly rinsed with 20 mM Tris buffer solution. The polymer pieces were transferred to another vial to be rinsed three times before they were taken out and examd under the microscope (Figure 3-20). The polymer pellets were taped onto the sample slide with double sided tape and insert into the instrument. Sinapinic acid (SA) was used on the polymer as the matrix. The cocaine retaining-aptamer modified RF polymer showed cocaine signal ($m/z$ 304) with SA as matrix (Figure 3-21). The 2-D and 3-D image of the intensity of the cocaine ($m/z$ 304) on the RF polymer is shown in Figure 3-22. The amount of the cocaine detected on the aptamer-indirect modified polymer by the MS is substantially higher than that on the direct
modified polymer (more spots with cocaine) due to the expected higher reaction efficiency of the indirect modification.

Conclusion

With the phenol groups on the RF polymer, it is relatively straight-forward to follow the established procedure to attach aptamer directly onto the RF polymer to surface modify it. The up-take of reactive green 19 and cocaine proved the existence of the aptamers on the RF polymer and the affinity capture capability of the aptamers.

With the phenol groups on the RF polymer, it is easy to follow the established procedure to attach carboxylic acid groups onto the RF polymer, then react with amine modified aptamers to surface modify the RF polymer with aptamers. The carboxylation of the RF polymer was proved by the detection of the up taking of toluidine blue O and PDAM. The amount of the cocaine detected on the aptamer indirect modified polymer by the MS is substantially higher than that on the direct modified polymer due to the expected higher reaction efficiency of the indirect modification.

With the procedure of attaching the aptamers onto the RF polymer established in the research project, other disease biomarker retaining-aptamers could be attached onto the RF polymer following the same procedure, which can be used for pre-analysis clean-up for MALDI-MS analysis for disease screening.
Figure 3-1. The structure of the RF polymer.
Figure 3-2. Chemical structure of reactive green 19. [Adapted from McGettrick, A.F.; Worrall, D.M. Protein purification protocols, 2nd edition *Methods in molecular biology*, vol 244, p 151 Edited by Cutler, P. Humana Press Inc., Totowa, NJ.]
Figure 3-3. The 27-mer oligodeoxyribonucleotide sequence, from $a$ to $b$ is designed the *consensus* sequence. [Adapted from Yılmaz, M; Bayramoğlu, G.; Arica, M.Y. Separation and purification of lysozyme by Reactive Green 19 immobilised membrane affinity chromatography *Food Chemistry* **2005**, *89*, 11]
Figure 3-4. The two cocaine isomers: a) pseudococaine and b) cocaine.
Figure 3-5. Anti-cocaine aptamer **MNS-4.1** bound to cocaine 1 (black ellipsoid). The bold region was cut out to generate **MNS-7.9**. Also shown are cocaine metabolites benzoyl-ecgonine 2 and eegonine methyl ester 3. [Adapted from Hermann, T.; Patel, D.J. Biochemistry - Adaptive recognition by nucleic acid aptamers *Science* **2000**, 287, 820]
Figure 3-6. The picture of a Applied Biosystems 3400 DNA Synthesizer.
Figure 3-7. Scheme of the indirect surface modification of the RF polymer via the carboxylation.
Figure 3-8. Chemical structure of PDAM.
Figure 3-9. Fluorescent emission intensity of PDAM-bond (blue) and noncarboxylated (red) RF polymer.
Figure 3-10. Structure of Toluidine blue O.
Figure 3-11. UV absorbance of the toluidine blue O desorbed from the carboxylated (red) and noncarboxylated (blue) RF polymer.
Figure 3-12. UV of the toluidine blue O solutions of different concentrations (1.0 μM, 2.0 μM, 3.0 μM, 4.0 μM, 5.5 μM, 7.3 μM, 9.1 μM, 14.8 μM).
Figure 3-13. Calibration curve of the concentration of the toluidine blue O solution at 285 nm. The imprecision of the concentrations of the toluidine blue O solutions caused the calibration curve not to pass the (0, 0) point.
Figure 3-14. UV absorbance of reactive green 19 desorbed from aptamer attached RF polymer (blue) and RF (red) polymer.
$y = 0.00061x + 0.00187$

$R^2 = 0.97734$

Figure 3-15. Calibration curve of the concentration of the reactive green 19 solution at 634 nm. The imprecision of the concentrations of the toluidine blue O solutions caused the calibration curve not to pass the (0, 0) point.
Figure 3-16. Cocaine (1) displaces diethylthiatricarbocyanine iodide complexed with aptamer MNS-4.1, causing an immediate attenuation of absorbance and eventual precipitation of dye. [Adapted from Stojanovic, M.N.; Landry, D.W. Aptamer-Based Colorimeter probe for cocaine *Journal of the American Chemical Society* **2002**, *124*, 9678]
Figure 3-17. A microscopic image of the indirectly cocaine retaining-aptamer modified RF polymer.
Figure 3-18. MS spectrum of the cocaine on A) the unmodified RF polymer. B) on the cocaine retaining-aptamer attached RF polymer.
Figure 3-19. The 2-D and 3-D image of the cocaine (m/z at 304) signal intensity across the indirect cocaine retaining-aptamer modified RF polymer. A) 2-D image. B) 3-D image.
Figure 3-20. The image of the direct cocaine retaining-aptamer modified RF polymer (3 mm in diameter).
Figure 3-21. MS spectrum of the cocaine on the direct cocaine retaining-aptamer modified RF polymer.
Figure 3-22. The 2-D and 3-D image of the cocaine signal intensity across the direct cocaine retaining-aptamer modified RF polymer. A) 2-D image. B) 3-D image.
CHAPTER 4
CONCLUSION AND FUTURE WORK

As a relatively new technology developed for the analysis of biomolecules, MALDI is very powerful; nevertheless, there is problems associated with the sample preparation methods and still room for the improvement of the spectral quality. The problems include the background noise from the matrix molecules which are used to assist in the ionization of the analytes in MALDI, and the uneven distribution of the analyte signal across the sample due to the inhomogeneous morphology of the matrix-analyte co-crystals associated with the traditional MADLI sample preparation methods. The complexity of biological samples also poses problem for the detection of the analyte of interest with low concentration. To address the problems facing MALDI-MS, an RF polymer and matrix-embedded RF polymers were synthesized and a direct and an indirect surface modification of the RF polymer with aptamers were developed.

An RF polymer and matrix-embedded RF polymers were synthesized via the sol-gel method. With the 1, 4 polymerization position on the aromatic ring, RF polymers can withstand the washing with aqueous solution, which make them suitable for analysis of biological sample and harsh condition of the surface modification with functional groups and aptamers; thus, they are a perfect substrate for MALDI and SELDI.

With the aromatic groups in the RF polymers and the reaction sites on the aromatic rings, it is possible to incorporate aromatic group-containing matrix molecules in the polymeric network with covalent bonds. Being trapped by the polymeric network with covalent bonds, the matrix molecules still provided the assistance for ionization, but dramatically reduced the amount of matrix molecule ablated and ionized by the laser due to the higher energy required to break the covalent bond than intermolecular interaction such as hydrogen bond, dipole-dipole interaction,
and van der Waals force. With much less matrix released and ionized, there is much less interference from matrix molecules in spectrum for low molecular weight analytes.

By incorporating the matrix molecules into the polymeric network structure, the problem of unevenly distributed signal across the sample due to the inhomogeneous morphology of the matrix-analyte co-crystals was solved. Many modifications of the traditional MALDI sample preparation methods have been developed to minimize the problem. Those modifications can more or less improve the evenness of the distribution of the analyte signal across the sample, but cannot fundamentally solve this problem. The new matrix-embedded resorcinol-formaldehyde (RF) polymers developed have the matrix molecules distributed in the polymer evenly at the molecular level, and trap the matrix molecules inside the polymer structure, which makes the formation of the matrix-analyte co-crystals unnecessary; thus, the evenness of the signal distribution only depends on the evenness of the analyte distribution. By using the most widely used and most problematic MALDI sample preparation method, dried-droplet, for both analyte-matrix solution on the stainless steel and analyte solution on the CHCA-embedded RF polymer, the reproducibility of analyte signal distribution was improved from 228% to 103% in RSD. This property also provides the possibility for quantization of the analyte in MALDI, which is to be evaluated in future.

With the phenol groups on the RF polymer, it is easy to follow established procedures to attach aptamer directly onto the RF polymer in a nucleic acid synthesizer. The aptamers attached onto the RF polymer are the reactive green 19-retaining aptamer and the cocaine-retaining aptamer. The uptake of reactive 19 by the aptamer modified RF polymer was detected by UV–vis spectrometry for the reactive green 19 desorbed from the reactive green 19-absorbed RF polymer. The amount of the reactive green 19 captured on the modified RF polymer was
determined according to the calibration curve constructed with the UV absorbance of toluidine blue O solutions with different concentrations. The uptake of cocaine by the aptamer-modified RF polymer was visually detected by replacing the aptamer affinity captured diethylthiatricarbocyanine iodide with cocaine. The cocaine captured RF polymer was analyzed in the LTQ MS with the detection of the cocaine signal ($m/z$ 304).

With the phenol groups on the RF polymer, it is also easy to follow established procedures to attach carboxylic acid groups onto the RF polymer. The carboxylation of the RF polymer was proved by the UV–vis detection of the uptake of toluidine blue O and PDAM. The amount of the carboxylic acid groups on the RF polymer was determined by the amount of the toluidine blue O desorbed from the toluidine blue O-absorbed RF polymer according to the calibration curve constructed with the UV absorbance of toluidine blue O with different concentrations. After the RF polymer was carboxylated, it was reacted with amine-modified cocaine retaining-aptamers to form the aptamer-modified RF polymer via the formation of an amide bond. The cocaine captured RF polymer was analyzed in the LTQ MS with the detection of the cocaine signal ($m/z$ 304) as done with the direct cocaine retaining-aptamer modified polymer. The amount of the cocaine detected on the aptamer indirect modified polymer by the MS is substantially higher than that on the direct modified polymer due to the expected higher reaction efficiency of the indirect modification.

The research conducted in this project has proved that the RF polymer is a versatile substrate that can both be derived with matrix molecules to provide more even analyte signal distribution across the sample and be surface modified with highly selective affinity binding aptamers for affinity capture prior to MS analysis. To fully assess the potential of the RF polymer, more research should be performed both on providing more even signal distribution
across the sample with matrix-embedded RF polymer and affinity capturing capability of the aptamer-modified polymer towards cocaine and expanding the the ranges of aptamers can be attached for affinity capture.

Other sample preparation methods such as airbrush or electrospray can be used to produce more even distribution of the analyte on the RF polymer to obtain more improved results and assess the detection limit of the analyte on the CHCA-embedded RF polymer. The assessment of the selectivity of the cocaine aptamer towards cocaine can be performed by affinity capture of cocaine from a mixture of cocaine and its metabolites solution and from biological samples such as blood and urine from a cocaine user. The lower limit of the affinity capturing ability of the specific cocaine-retaining aptamer also needs to be tested, since the concentration of cocaine solution employed here for affinity capturing is 30 ppm. The concentration of unmetabolized cocaine extracted in urine is much lower; indeed, the concentration of extracted cocaine is one hundredth of the concentration of the cocaine metabolites (1 ppm).\textsuperscript{141} The affinity capturing of the cocaine with the aptamer used in this experiment may not possess the detection limit needed for the analysis of urine samples from individuals using cocaine because of the inherent high dissociation constant (0.4-10 $\mu$M) between the aptamer and cocaine. The dissociation constant between an aptamer and the target should be around $10^{-15}$ M.\textsuperscript{143} A different cocaine aptamer with lower dissociation constant should be synthesized by SELEX process for higher selectivity and then be attached onto the RF polymer following the procedure established in this research. Aptamers with high affinity toward disease biomarkers can also be synthesized by SELEX process and be attached onto the RF polymer following the same indirect modification procedure. Then these disease biomarker-retaining aptamer modified RF polymers can be used
to affinity capture the disease biomarkers from biological samples taken from individuals for pre-analysis clean-up for MALDI-MS analysis for disease screening.
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BIOGRAPHICAL SKETCH

Hong Yu was born in Shanghai, People’s Republic of China, on November 6th, 1967, to Shunyang Yu and Huaizhen Yan. Hong graduated from Shanghai Middle School in 1986 and went to East China University of Science and Technology, where she majored in inorganic nonmetallic materials. After receiving a Bachelor of Science degree in inorganic nonmetallic materials at age 23, Hong began work at Shanghai TV Glass Tube Plant as a ceramic engineer, and stayed there for ten years till the plant was shut down. Hong then started a job at Shanghai Omniworks Electronics Ltd. as a QA engineer. In August 2000, Hong came to United States of America to attend the master’s program in chemistry at Southern Illinois University at Edwardsville to work under the tutelage of Professor Timothy Patrick. She received a Master of Science in chemistry in August of 2003; she graduated with high honors and received an award as an outstanding graduate student. Hong also met her future husband at Southern Illinois, James Rogers, who also received a Master of Science in Chemistry in the summer of 2003. In the summer of 2002, Hong and James began their PhD study at the University of Florida and were married in 2003. Hong is working under the mentorship of Professor Richard Yost. James obtained his PhD. degree in December 2005 and took on the job as a patent examiner at US Patent and Trademark Office at Washington DC in January 2006. Hong suspended her study in the PhD program and moved to Washington DC with James and their daughter, Elaine Rogers, who was born on May 22, 2005. In August 2006 Hong brought their daughter along with her back to the University of Florida to continue her study in PhD program in Chemistry.