Characterization of native and bypass human coronary artery plaque deposits from the same heart: investigation of the chemical form of calcium in human coronary artery plaque deposits

Serigne Thiam
Louisiana State University and Agricultural and Mechanical College, sthiam@lsu.edu

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CHARACTERIZATION OF NATIVE AND BYPASS HUMAN CORONARY ARTERY PLAQUE DEPOSITS FROM THE SAME HEART: INVESTIGATION OF THE CHEMICAL FORM OF CALCIUM IN HUMAN CORONARY ARTERY PLAQUE DEPOSITS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agriculture Mechanical College
In partial fulfillment of the
Requirement for the degree of
Doctor of Philosophy

In

The Department of Chemistry

By
Serigne Thiam
B.S. University Cheikh Anta Diop of Dakar (Senegal), 1985
M.S. Pennsylvania State University, University Park, PA, 1997
August, 2003
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<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CEC</td>
<td>capillary electrochromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>MEC</td>
<td>micellar electrokinetic chromatography</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Poly-SUG</td>
<td>polymerized sodium N-undecenoyl sulfate</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micelle concentration</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>Tris</td>
<td>tris[hydroxymethyl]aminoethane</td>
</tr>
<tr>
<td>EOF</td>
<td>electroosmotic flow</td>
</tr>
<tr>
<td>APCI</td>
<td>atmospheric pressure chemical ionization</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electro spray ionization-mass spectrometry</td>
</tr>
<tr>
<td>TOF</td>
<td>time-of-flight mass spectrometry</td>
</tr>
<tr>
<td>GC/MS</td>
<td>gas chromatography/mass spectrometry</td>
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<tr>
<td>ICP-MS</td>
<td>inductively coupled plasma mass spectrometry</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>carbon-13</td>
</tr>
<tr>
<td>$^{31}$P</td>
<td>phosphorus-31</td>
</tr>
<tr>
<td>$^1$H</td>
<td>proton</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CH</td>
<td>methane</td>
</tr>
<tr>
<td>CH$_2$</td>
<td>methylene</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>methyl</td>
</tr>
<tr>
<td>CP</td>
<td>cross polarization</td>
</tr>
<tr>
<td>MAS</td>
<td>magic angle spinning</td>
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<tr>
<td>ppm</td>
<td>parts per million</td>
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<tr>
<td>XANES</td>
<td>x-ray absorption near edge spectroscopy</td>
</tr>
<tr>
<td>EXAFS</td>
<td>x-ray absorption fine structure</td>
</tr>
<tr>
<td>XPS</td>
<td>x-ray photoelectron spectroscopy</td>
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<tr>
<td>SR-XMT</td>
<td>synchrotron radiation x-ray micro-tomography</td>
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TERMINOLOGY

**Aorta**
The main blood vessel that carries oxygenated blood away from the heart to the body

**Vein**
Blood vessels that transport oxygen-poor but, pulmonary blood away from the body towards the heart

**Artery**
Blood vessels that carry oxygen but, pulmonary blood away from the heart to the body

**Angina**
Severe constricting pain that patients with heart disease often experience (e.g. when over-exerting themselves)

**Angiogram**
Radiographic image of blood vessels in a living patient

**Angioplasty**
Procedure to open blocked blood vessel

**Atherosclerosis**
-Multi stage process
Hardening of change overtime sized arteries (characteristically irregular distribution of lipid deposits in arterial intima. Then deposits are covered within fibrous cap.

**Calcification**
Deposition of calcium. in the arterial wall

**Stenosis**
Blockage, obstruction, or narrowing of the arteries

**Hemorrhage**
Bleeding

**Thrombosis**
Formation of blood clots inside of a blood vessel

**Lymphocytes**
Lymphocytes are types of white blood cells or leukocytes formed in lymphatic tissue. They are extraordinarily diverse in their functions. The most abundant lymphocytes are: B lymphocytes (B cells) and T lymphocytes (T cells).
Macrophage
Large scavenger cell present in connective tissue and many organs and tissues

Endothelium
The endothelium is located at the interface between the blood and the vessel wall

Acyl-CoA cholesterol acyltransferase (ACAT)
The enzyme that esterifies cholesterol for storage

Osteopontin (OPN)
OPN is an acidic protein, with several Asp or Glu residues, which exhibits a high amino acid homology between species (mouse, rat, human and pig). The protein is associated with mineralized ion and contains calcium or hydroxyapatite-binding site.

Osteonectin
A protein that plays role in mineralization of covalently binds bone

Osteocalcin
A bone matrix protein

Matrix Gla protein (MGP)
The protein controls the formation and the restoration of bone. The protein has strong affinity for calcium binding and can be crystallized in the form of hydroxyapatite.

Raised lesion
Raised lesion is characterized by the presence of two components in the plaque: the fibrous cap and the atheroma.

Fibrous cap
The fibrous cap is a layer of fibrous connective tissue, which is thicker and less cellular than the normal intima. The fibrous contains macrophages and smooth muscle cells.

Atheroma
The atheroma consists of a mass of necrotic lipid. Usually, the term refers to the mass of fatty materials, but sometimes is used to describe advanced plaque deposits with lesions.

Fatty streak
Flat or slightly elevated lesions that contain intracellular and extracellular lipids in intima. Fatty streaks are localized in the early stages of atherosclerosis

Crystalline deposits or hard plaques
Mass of organic and inorganic materials in the crystalline phase of the plaque deposits

Soft plaque
Smooth and fatty deposits, sometimes called amorphous plaque
## Terms Used in Pathology to Define the Types of Human Atherosclerotic Plaque Lesions

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<th>Atherosclerotic Lesions</th>
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<td>Type I</td>
<td>Initial lesion</td>
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| Type II | Progression-prone type II lesion  
Progression-resistant type II lesion | Fatty dot or streak  
Early lesions |
| Type III| Intermediate lesion (preatheroma) |                                    |
| Type IV | Atheroma                 | Atheromatous plaque, fibrolipid plaque, fibrous plaque, plaque Calcified plaque  
Advanced lesions, raised lesions |
| Type V  | Fibroatheroma (type V lesion)  
Calcific lesion (type VII lesion)  
Fibrotic lesion (type VIII lesion) | Fibrous plaque  |
| Type VI | Lesion with surface defect, and/or hematoma-hemorrhage, and/or thrombotic deposit | Complicated lesion, complicated plaque  |

(1998 American Heart Association)
ABSTRACT

Gradual deposits of lipids, proteins, and calcium on the coronary arterial walls cause atherosclerosis, leading to blockage of the blood flow and possible heart attack. Despite many studies, the mechanism underlying these processes remains unclear. In this research, differences between native and bypass plaque deposits from the same heart were examined. In addition, the crystalline and the amorphous plaques within these native and bypass vessels were characterized. The techniques used to characterize the deposits included inductively coupled plasma mass spectrometry (ICPMS), solid-state nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC), etc.

The results from the ICPMS technique showed that the concentrations of Ca and P varied widely in the bypass arteries. In contrast, the concentrations of Ca and P were higher in the native deposits. The molar ratios of Ca/P indicated that these elements probably exist as hydroxyapatite in the calcified tissues. However, the molar ratio of Ca/P in the soft tissue suggests that the phosphorus does not only exist as hydroxyapatite but that at least half of it may also be organic.

The $^{13}\text{C}$ and $^{31}\text{P}$ solid-states NMR spectra of the native and bypass coronary plaques from the same heart indicated that the concentrations of carbonyl groups, typical of cholesterol esters, were similar between the native and bypass arteries. Significant signals of carbonyl groups in the crystalline and amorphous plaques were due to amino acids and proteins deposited in the plaques. Studies of $^{31}\text{P}$ showed that the phosphorus exists mostly as hydroxyapatite in the crystalline native plaque, but a large proportion exists as organic phosphorus.
To study the interactions of calcium with homocysteine and cholesterol, $^{13}$C solid-state NMR of homocysteine, Ca-homocysteine, Ca-homocysteine-cholesterol, Ca-cholesterol were performed. Significant spectral changes were also noted when calcium was added to homocysteine and cholesterol.

The chemical forms and the distribution of calcium were studied using x-ray absorption and light microscopy with silver staining. Finally, chromatographic methods (CEC and APCI-MS) showed that only 40% or less of the plaque in native or bypass arteries consisted of cholesterol and its esters and the remaining, 60% were composed of proteins, fatty acids, and phospholipids.
Atherosclerosis

The accumulations of organic and inorganic materials in human coronary arteries result in the formation of plaque deposits. Over time plaques continue to deposit on the arterial wall reducing the artery diameter. Thus, there is a decrease in the blood flow, which may eventually cause a heart attack or stroke. This process of plaque formation is called atherosclerosis [1-9]. However, the mechanism responsible for plaque formation is not well understood. Figure 1.1A shows a cross section of a normal artery. The blood flows through the lumen. Then, plaque deposition starts to occur due to the dysfunction of the endothelium of the arterial walls. Then lipoproteins become trapped in the arterial wall, and monocytes, and lymphocytes adhere to the artery surface. Eventually, the structural formation of the plaque deposits involves the lymphocytes forming initial lesions known as fatty streaks (Figure 1.1B). Fatty streaks are deposits of intracellular (foam cells) and extracellular lipids found in the intima. Gradually in the connective tissue, smooth muscle cells form, which include fiber proteins, collagen, and proteoglycans. Over time the cell components diffuse leading to the transformation of monocytes to macrophages. In the later stage of the disease, more lipids and cholesterol (free and esterified cholesterol) continue to accumulate on the arterial wall (Figure 1.1C). In summary, the lesions in the early stages are known as fatty streaks and the later stages are known as fibrous plaques [1-9].
Figure 1.1  A) Cross section cut of a normal artery.  B) Early stage of atherosclerotic plaque deposits. There is a proliferation of smooth cells; and presence T cells platelets, and leucocytes. These events are indicators of atherosclerosis. C) Schematic of advanced deposits with accumulation necrotic core, necrotic caps, and macrophages [2].

There is a growing consensus that molecules, other than cholesterol, may enhance the plaque formation, destabilize the existing plaques, or trigger inflammation and degeneration of the arterial walls [10-17]. These advanced plaques have been associated with high levels of cholesterol, cholesteryl esters and calcium [6-10]. For example, Hirsch et al. revealed that in human atherosclerotic lesions, unesterified cholesterol and calcium phosphate, in the form of hydroxyapatite, are in close proximity to each other within the same plaque deposit [18]. Currently, it is not obvious whether cholesterol
induces calcium deposition or vice versa, because in advanced plaque deposits, calcium complexes and cholesterol derivatives are present in large crystalline aggregates [18].

**Risk Factors**

Homocysteine, an amino acid, when present in the blood contributes to endothelium damage, harms smooth muscle of the arterial walls, and enhances plaque formation. In 1969, McCully proposed that the homocysteine concentrations in the blood are proportional to the risk of heart disease [19]. To explain the cause of injuries in the arterial walls, Wang, *et al.* elaborated on McCully’s homocysteine medical hypothesis [20]. Wang’s hypothesis indicates that a high presence of homocysteine in the blood enables the formation of calcium/homocysteine related products [20]. High concentrations of homocysteine in the blood favor interactions with calcium ions of the calcium-dependent cell junction [20]. The calcium/homocysteine interaction causes structure dissociation, which can damage the vascular endothelium. The levels of homocysteine in the body are influenced by diet, smoking, genetics, drugs, menopause, exercise, and blood pressure [21-23].

Exposure to tobacco smoke also has a detrimental effect on heart disease [24-26] by enhancing lesion formation. It is believed that free radicals in cigarette smoke cause oxidation of lipids [27]. Previous studies comparing the susceptibility of lipid peroxidation in smokers and non-smokers have been inconsistent [27]. One explanation is the dietary difference between smokers and non-smokers. The dietary and serum levels of antioxidants are lower in smokers than nonsmokers. Hence, the susceptibility of lipid peroxidation in smokers depends on the levels of serum antioxidants. Miller *et al.* demonstrated that cigarette smokers have higher degrees of lipid peroxidation than
nonsmokers [30,31]. In addition, the Pathobiological Determinant Research Group concluded that the serum thiocyanate concentration, a marker for smoking, was found in high concentrations in plaque deposits.

The body naturally produces cholesterol for cell function, and lipoproteins assist in the transportation of cholesterol into the blood stream. Lipoproteins include very low-density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low-density lipoprotein, and high-density lipoprotein (HDL). These lipoproteins have very complex structures where the protein portion can be very diverse in structure and the lipid portions have the composition of cholesterol and cholesteryl esters. HDL and LDL are the most important lipoproteins because of their roles in the biological system. HDL, the so-called “good” cholesterol, primarily transfers cholesterol back to the liver where it is transformed into bile acids. However, LDL transports cholesterol from the liver to the rest of the body and helps to facilitate lipid uptake by the cells. When LDL is degraded in the cells, the protein portion is hydrolyzed to cholesterol and fatty acids to supply the cellular membranes. The excess of cholesterol is then stored as cholesteryl oleate or cholesteryl palmitate. These esters are synthesized by the process of acyl-CoA cholesterol acytransferase (ACAT), which is enhanced by the presence of free cholesterol [1, 32].

Calcification of the Arterial Wall

Vascular calcification is associated with the deposition of calcium phosphate complexes (especially hydroxyapatite) in coronary tissues. It is well established that calcification is one of the main characteristics of advanced atherosclerosis. Calcium deposits in the arterial walls can be enhanced when the patient is diabetic or has some
renal dysfunction. Recent studies have revealed that calcification is considered to be the final stage of atherosclerosis progression [6-8]. Despite studies that are involved in the characterization of complex proteins associated with vascular calcification, such as osteopontin, osteonectin, esteocalcin, and matrix Gla protein (MGP), the mechanism controlling the calcification of tissues remains unclear. However, there is some evidence that serum phosphate levels are linked to calcification of tissues. Clinical studies have shown that phosphate serum levels at higher-than-normal concentrations (1.0 to 1.5 mmol/L) correlate with vascular calcification. In those cases, most of the vascular calcification occurs as a result of a mineral imbalance. Giachelli, et al, tested this hypothesis by monitoring the mechanism controlling vascular calcification [33,34]. Their finding suggested that human aortic smooth cells (HSMC) calcification was controlled at least in part through a sodium-dependent phosphate transport-sensitive process. This kind of procedure characterized the response of HSMC after they had been exposed to high phosphorus levels.

Over the years, calcium and vitamin D have been prescribed to enhance deposition of calcium in bones. However, the intake of the osteoregulatory molecules may also have an impact on the calcium deposition in vascular walls [35]. To assess the potential of osteo-regulation on the development of vascular calcification, Watson, et al. investigated the vitamin D levels in serum in association with vascular calcification [35]. They found that vitamin D was inversely correlated with the progression of vascular calcification in both populations. Furthermore, no correlation was found between the extent of calcification and levels of osteocalcium and parathyroid hormones.
Proposed Treatments of Blocked Arteries

Atherosclerotic blockages of the coronary arteries can cause angina or heart attacks. Usually, when the medication or the changes of lifestyle (diet, exercise, and smoking) do not improve the heart health, the physician may recommend angioplasty or a coronary artery bypass surgery. Due to the risks of these medical interventions, doctors reserve angioplasty or bypass operation for patients who do not derive the maximum benefit from medication [36,37].

In coronary angioplasty, a catheter with a small balloon on the tip is inserted into an artery either in the groin or the arm. Then, the catheter is pushed into the coronary arteries and the balloon tip is enlarged in order to open the blockage. This procedure is called percutaneous transluminal coronary angioplasty (PTCA). PTCA is not very efficient for removing serious blockages [36,37]. Bypass surgery is an alternative procedure to treat blocked arteries. Coronary artery bypass grafting involves the removal of a healthy saphenous vein from the leg of the patient for use as the bypass artery. One end of that vein is connected to the aorta, near the origin of the blocked coronary artery [36-39]. Then, the other end is connected to the lower end of the same coronary artery. A similar bypass procedure can be performed using the internal mammary arteries instead of the saphenous veins. Figure 1.2 is an illustration of a bypassed artery around the blockage.

Although surgery may improve the heart conditions of patients, it is not clear why about 40% of people who had bypass operations show symptoms of a new blockage either in the bypass grafts or in another coronary artery within 10 years of the operation. Furthermore, complications such as angina or death can occur in people with coronary
bypass grafts [37-39]. Because of all the risks that can occur during or after the operation, it is crucial to gain more understanding of the formation mechanism of the coronary artery blockages.

![Illustration of two bypassed arteries around the blockages](image)

**Figure 1. 2 Illustration of two bypassed arteries around the blockages [36].**

**Part II. Background on the Analytical Methods Used in this Dissertation**

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

Inductively coupled plasma mass spectrometry (ICP-MS) determines the concentrations of trace metals [40-43]. ICP-MS provides minimum interferences and can be applied in a wide range of studies. In addition, ICP-MS can be coupled with separation instruments, such as high performance liquid chromatography (HPLC), capillary electrophoresis (CE), and capillary electrochromatography (CEC). While ICP-
MS is not the only available technique for metal analysis, it is sensitive to a wide group of metals.

**Inductively Coupled Plasma (ICP) Source**

Plasma is an electrically neutral gas, which is composed of free moving electrons and ions [40-48]. A monatomic gas in a plasma state can be described by use of the following equation [40-48]

\[
X = \sum_{n=1}^{q} X^{n+} + \sum_{n=1}^{q} n(e^-),
\]

(1.1)

where \(X^{n+}\) is the ionic form with \(n\) charges and \(e^-\) the charge of an electron.

---

**Figure 1.3** A) Inductively coupled plasma source. B) Inductively coupled plasma torch.
For ICP, plasma is generated when argon flows through a radio frequency field. Then, the argon plasma passes through a quartz tube, which has a 1 to 25 kW radio frequency power source. Figure 1.3 has a schematic of an ICP torch [48]. When the sample atoms reach the top of the source, they are exposed at temperatures ranging from 4000 to 8000 K. These temperatures are enough to allow complete atomization and ionization interference effects. The ionization of the plasma gas is ionized when electrons and the argon molecules collide [46-70], i.e.,

\[ e^- + Ar = Ar^+ + e^- + e^- \]  \hspace{1cm} (1.2)

where \( Ar \) is the argon molecule, \( Ar^+ \) is the argon ion, and \( e^- \) is the charge of an electron.

Then, through a recombination of processes, argon ions recombine with electrons to form an excited argon molecule, \( Ar^* \) [46-70], i.e.,

\[ e^- + Ar^+ = Ar^* + h\nu \]  \hspace{1cm} (1.3)

Finally, analytes are ionized as a result of electronic interaction between species in the plasma. Then, analyte ions are measured using mass spectrometry.

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

In ICP-MS, samples are injected into the center of the plasma as aerosols. The ICP plasma source dissociates the sample into its elemental composition. Then, the ions migrate into the mass spectrometer, where they are separated based on their mass-to-charge ratio, \((m/z)\) [71-80]. Figure 1.4 is a representation of an ICP-MS design. In most cases, a quadrupole or magnetic sector mass analyzer is used to determine the \(m/z\) ratios of the ion abundance.
The use of ICP-MS offers several advantages. It is rapid, and it allows simultaneous multi-element measurement at low detection (ppb or less for some elements). In addition, ICP-MS also has relatively small matrix effects with a wide dynamic range (up to 5–6 orders of magnitude), a precision ranging from 0.5 to 5%. ICP-MS can be applied to gases, liquids, and solids [71-80]. However, this technique has a few problems related to spectral interferences. Also, the analysis of solid samples can be a challenge [72-74]. Such effects are more pronounced for the elements like calcium (Ca) and sulfur (S). Therefore, the low limit of detection for elements like Ca, iron (Fe), and S are usually affected [80]. Table 1.5.1 is an illustration of typical limits of detection for different elements obtained in an ICP-MS analysis. Table 1.5.2 lists some examples of spectral overlap interferences encountered with ICP-MS [76].
Table 1.5.1 Limits of detection* for some biologically relevant elements by the use of the most abundant isotope in ICP-MS [80]

<table>
<thead>
<tr>
<th>Analyte</th>
<th>ICP-MS (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al</td>
<td>0.05</td>
</tr>
<tr>
<td>As</td>
<td>0.01</td>
</tr>
<tr>
<td>Ba</td>
<td>0.001</td>
</tr>
<tr>
<td>Cd</td>
<td>0.005</td>
</tr>
<tr>
<td>Ca</td>
<td>0.5</td>
</tr>
<tr>
<td>Cu</td>
<td>0.005</td>
</tr>
<tr>
<td>Cr</td>
<td>0.005</td>
</tr>
<tr>
<td>Fe</td>
<td>0.1</td>
</tr>
<tr>
<td>Pb</td>
<td>0.001</td>
</tr>
<tr>
<td>Mg</td>
<td>0.05</td>
</tr>
<tr>
<td>Hg</td>
<td>0.001</td>
</tr>
<tr>
<td>K</td>
<td>0.5</td>
</tr>
<tr>
<td>P</td>
<td>1.0</td>
</tr>
<tr>
<td>Na</td>
<td>0.05</td>
</tr>
<tr>
<td>S</td>
<td>50</td>
</tr>
<tr>
<td>Zn</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* The limit of detection (LOD) is the lowest amount that can be detected with precision using a given method.
Table 1.5.2 Examples of spectral overlap interferences encountered with ICP-MS [74]

<table>
<thead>
<tr>
<th>Ion</th>
<th>Mass</th>
<th>Source</th>
<th>Interferes with elements of interest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar&lt;sup&gt;+&lt;/sup&gt;</td>
<td>36,38,40</td>
<td></td>
<td>Ca S K</td>
</tr>
<tr>
<td>ArH&lt;sup&gt;+&lt;/sup&gt;</td>
<td>37,39,41</td>
<td>Plasma gas</td>
<td>K Cl</td>
</tr>
<tr>
<td>Ar&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>76,78,80</td>
<td></td>
<td>Ge Se Kr</td>
</tr>
<tr>
<td>Ar&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>18,19,20</td>
<td></td>
<td>O F</td>
</tr>
<tr>
<td>ArO&lt;sup&gt;+&lt;/sup&gt;</td>
<td>52,54,56</td>
<td></td>
<td>Fe Cr</td>
</tr>
<tr>
<td>OH&lt;sub&gt;n&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>16,17,18,19</td>
<td>Water</td>
<td>O F</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;n&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>32,33,34</td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>N&lt;sup&gt;+&lt;/sup&gt;</td>
<td>14,15</td>
<td>Nitric acid</td>
<td>N</td>
</tr>
<tr>
<td>NO&lt;sup&gt;+&lt;/sup&gt;</td>
<td>30,31,32,33</td>
<td>Matrix, traces</td>
<td>P S Si</td>
</tr>
<tr>
<td>N&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt;</td>
<td>28,29,30</td>
<td>in plasma gas</td>
<td>Si</td>
</tr>
<tr>
<td>C&lt;sup&gt;+&lt;/sup&gt;</td>
<td>12,13</td>
<td>Traces in plasma gas</td>
<td>C</td>
</tr>
<tr>
<td>ArCl&lt;sup&gt;+&lt;/sup&gt;</td>
<td>75,77</td>
<td>Hydrochloric acid matrix</td>
<td>As Se</td>
</tr>
<tr>
<td>ClO&lt;sup&gt;+&lt;/sup&gt;</td>
<td>51,53</td>
<td>acid matrix</td>
<td>V Cr</td>
</tr>
<tr>
<td>CuCl&lt;sup&gt;+&lt;/sup&gt;</td>
<td>98,100,102</td>
<td></td>
<td>Mo Ru Pd</td>
</tr>
<tr>
<td>Ba&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>65,66,67,671/2,68</td>
<td></td>
<td>Cu Zn Ga</td>
</tr>
<tr>
<td></td>
<td>681/2,69</td>
<td>If M&lt;sup&gt;+&lt;/sup&gt; present at high concentration</td>
<td></td>
</tr>
<tr>
<td>Th&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>116</td>
<td></td>
<td>Cd Sn</td>
</tr>
<tr>
<td>U&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>119</td>
<td></td>
<td>Sn</td>
</tr>
<tr>
<td>2Mg&lt;sup&gt;+&lt;/sup&gt;</td>
<td>48,50,52</td>
<td></td>
<td>Ti Ca V Cr</td>
</tr>
</tbody>
</table>

Underlined mass numbers are the most abundant isotopes of the element.
Nuclear Magnetic Resonance

Background

The discovery and applications of NMR are marked by a number of very important events. In 1924, Pauli demonstrated that nuclei have an angular momentum [81]. In the same period, Gerlach and Stern determined the nuclear magnetic moments using atomic beam measurement [82]. In 1938, Retri and colleagues observed the resonance effect of nuclei when electromagnetic radiation was applied [83]. In 1946, after several years of exploration, Block at Stanford University and Purcell at Harvard University reported the first $^1$H NMR in liquid--H2O and solid paraffin wax [84]. In 1953, this work was rewarded with a Nobel Prize in Physics. Although NMR theory is relatively complex, the development of the technique seems to be more promising.

Since its discovery by Felix Bloch and Edward Purcell, NMR has progressed considerably [85-95]. For a number of years, proton NMR has been performed to determine the physical properties of liquid and solid samples [94]. At present, NMR experiments have been extended to a wide variety of nuclei due to the availability of more powerful FT methods. In an effort to increase the sensitivity of the technique to study biomolecular species, NMR methods, such as high resolution NMR and magic angle spinning NMR have been developed [95-97].

Spin Properties

NMR measurements involve the interactions of radiowaves and the spinning of precessing nuclei in a molecule [97,98]. Although the energy of radiowaves is small, the nuclear spin of atoms is still affected. Each atom is characterized by its spin number or angular momentum (Table 1.6.1). Even number of protons or neutrons result in a zero
spin number. In comparison, an odd number of protons or neutrons result in a non-zero spin number. For example, $^{12}$C has an atomic weight of 12 equivalent to the same number of protons and neutrons. Thus, the net spin is zero, and so is the angular momentum. The zero angular momentum of $^{12}$C is not amenable to NMR experiments. In contrast, the $^{13}$C has an even number of protons and an odd number of neutrons; therefore, its spin number is $\frac{1}{2}$ (where in this case $n=1$). Such a spin value allows $^{13}$C NMR to be performed. Other $\frac{1}{2}$ spin types of NMR include $^1$H-NMR, $^{31}$P-NMR, and $^{19}$F-NMR.

Table 1.6.1 Spin number of nuclei [97,98]

<table>
<thead>
<tr>
<th>Number of mass = atomic weight (Proton + Neutron)</th>
<th>Atomic Number (Proton)</th>
<th>Spin Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Odd</td>
<td>Odd or Even</td>
<td>$1/2$, $3/2$, $5/2$</td>
</tr>
<tr>
<td>Even</td>
<td>Even</td>
<td>0</td>
</tr>
<tr>
<td>Even</td>
<td>Odd</td>
<td>1, 2, 3</td>
</tr>
</tbody>
</table>

Nuclei with a non-zero spin quantum number, $I$, behave like a small magnet with magnetic moment, $\mu$, defined by the equation [90,91]

$$\mu = \frac{\gamma h [I(I + 1)]^{1/2}}{2\pi},$$  \hspace{1cm} (1.4)

where $h$ is the Planck’s constant and is $\gamma$ the magnetogyric ratio. The projection of $\mu$ along the z-axis, $\mu_z$, is given by

$$\mu_z = m\gamma h / 2\pi,$$  \hspace{1cm} (1.5)
where \( m_l \) is the magnetic quantum number with the allowed values ranging from \((I-1)\ldots0\), \((-I+1)\ldots-I\). Overall \(2I+1\) represents the possible states of the nucleus. In the absence of an applied magnetic field, \( m_l \) is independent of the energy of the nucleus. However, the degeneracy is cancelled when an external field is applied, and the energies of the nuclei are given by [92]

\[
E = -\mu_B B_0 = -m\gamma B_0 / 2\pi ,
\]

(1.6)

where \( B_0 \) is the applied magnetic field in the z-axis direction.

At spin \( 1/2 \) the nuclei are associated with two states corresponding to \( m_l = 1/2 \) and \(-1/2\). The difference of energy between the two states is [90,91]

\[
f_0 = \gamma B_0 / 2\pi ,
\]

(1.7)

where \( f_0 \) is the Larmor frequency which arises from the interaction of the spinning nucleus with the magnetic field causes the magnetic moment to precess at \( f_0 \).

**Mode of NMR**

During the first 25 years of its discovery, NMR experiments were usually performed using the continuous wave CW method. Currently; all modern NMR spectrometers operate in the pulse-FT mode. The CW method consists of either sweeping the magnetic field while holding the radio frequency constant or vice-versa. On the other hand in FT-NMR, a short RF pulse is applied as a result of the magnetization, and \( M_{xy} \) becomes nonzero [90-99]. \( M_{xy} \) processes around the z-axis at \( f_0 \) [90-99]. The nuclear magnetic moment precesses around \( B_0 \) with no phase coherence. Due to the population difference in energy states, there is a net-magnetization in the direction of \( B_0 \) along the z-axis. A nuclear induction signal from macroscopic magnetization in the
transverse (x,y) plain $M_{xy}$ is detected using the pulse/FT spectrometer. The value of $M_{xy}$ is zero at equilibrium.

In FT-NMR, a transient signal is induced by the precessing $M_{xy}$ in a receiver coil called the free induction decay (FID), which is orthogonal to the directions of $B_0$. Since the RF probe excites the nuclei over a wide frequency range, the FID illustrates the time domain signals from all the nuclei, which resonant in the given frequency range. Then, the Fourier transform of FID gives the frequency domain spectrum. In addition, multiple-pulse NMR was introduced in order to be able to manipulate the nuclear magnetization by RF pulses. Such methods allow the measurement of spin-lattice ($T_1$) and spin-spin ($T_2$) relaxation times for each resonance [100]. In addition, multiple pulse sequences can be used to increase the range of NMR applications. A diagram of an NMR spectrometer with its components is provided in Figure 1.5. The main component of an FT-NMR spectrometer is a magnet in which the sample is inserted. The receiver coil or transmitter surrounds the sample. The coil supplies the electromagnetic energy, which is necessary for the spin to change its orientation. The nuclei are excited using a pulse, which is provided by a controlled oscillator. This excitation of the nuclei is the result of energy absorption. Therefore, an amplified radiofrequency signal is induced. The signal is transmitted through a coil. Then, the signal, called free induction decay (FID), is amplified and transmitted to the detector. The detector computes the frequency difference between the two states of the nuclei. Then, the Fourier transform program is applied to the output in order to give a frequency domain spectrum.
Solid State NMR

Solid state NMR is a non-destructive technique, which is used to determine the structures of molecules in the solid state. Many theoretical aspects of liquid NMR remain applicable in solid state NMR. Solid and semisolid state NMR delivers lower resolution spectra than liquid state NMR. In solid state NMR, poor resolution and broadband are due to dipole-dipole interactions and chemical shift anisotropy [104]. For example the solid dipole-dipole interactions are static, and they are a major source of line broadening because the chemical shift anisotropic value is not averaged [104].

The lack of resolution can be explained using quantum chemical theories [105]. By considering the terms in the nuclear spin Hamiltonian, the cause of line broadening can be understood in the following equation [106].

\[ H = H_o + H_1 + H_2 + H_3 , \]  \hspace{1cm} (1.8)
where $H_{cs}$ is the chemical shift, $H_j$ is the scalar spin coupling term, $H_d$ is the dipolar coupling, and $H_q$ is the quadrupolar Hamiltonian. The values of each term in the nuclear spin Hamiltonian expression are reported in Table 1.7.1.

**Table 1.7.1 Nuclei spin Hamiltonian parameters** [106]

<table>
<thead>
<tr>
<th>Sample</th>
<th>$H_{cs}$</th>
<th>$H_j$</th>
<th>$H_d$</th>
<th>$H_q$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>$10^3$-$10^4$</td>
<td>$10$-$10^2$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Solid</td>
<td>$10^3$-$10^4$</td>
<td>$10$-$10^2$</td>
<td>$2$-$10^4$</td>
<td>$10^5$-$10^6$</td>
</tr>
</tbody>
</table>

Equation (1.8) contains 4 terms, and each of them depends on the magnitude of the dipole-dipole interactions in Hertz (Hz) for liquid and solids. For small molecules in liquid, the dipolar ($H_d$) and the quadrupolar ($H_q$) terms vanish due to the rapid isotropic molecular motion. [106]. The dipolar Hamiltonian for proton spin can be expressed as [106]

$$H_d = \gamma^2 h^2 \sum_{i,j} - \frac{1}{r_{ij}^2} (r_{i,j} - 3r_{i,z}r_{j,z}) (I_i I_j - 3I_{i,z}I_{j,z}), \quad (1.9)$$

where $r_{i,j}$ is the length of the internuclear vector, $I$ is the spin operator, $\gamma$ is the gyromagnetic ratio, and $\hbar$ is the Planck’s constant

$$r_{i,j} = r \cos \theta, \quad (1.10)$$

where $\theta$ is the angle between the internuclear vector and $B_0$.

$r_{i,j}$ from equation 1.10 can be substituted into the equation 1.9.
\[ H_d = \gamma^2 \hbar^2 \sum_{i,j} - \frac{1}{r^2 i,j} (3 \cos^2 \theta_{i,j} - 1)(I_i I_j - 3I_{i,z} I_{j,z}) \] (1.11)

In liquids, \( \theta_{i,j} \) is the function of rapid, tumbled, and isotopic effects. The angular factor in equation 1.11 should be determined as [106]

\[ \langle 3 \cos^2 \theta_{i,j} - 1 \rangle = \int_0^{2\pi} (3 \cos^2 \theta_{i,j} - 1) \sin \theta d\theta ; \] (1.12)

thus, \( \langle H_d \rangle = 0 \). The quadrupole Hamiltonian has the same value as the dipolar Hamiltonian; therefore, the spectra of solutions will depend only on the chemical shift and scalar spin coupling Hamiltonian \( (H_{cs} + H_J) \). In contrast, in the solid state, the system is rigid. As a result \( \theta_{i,j} \) is fixed with respect to \( H_o \) and \( H_d \) for two protons. The \( H_d \) and \( H_q \) are larger than \( H_{cs} \) and \( H_J \) in the solid state because the motion is strongly anisotropic. Therefore, the averaged \( H_d \) is incomplete. For both solids and semisolids the line is still broad.

Line broadening occurs in solid sample due to restricted motions. Thus, the dipolar couplings, \( D \), are not fully averaged to zero, and have a time-averaged dependence shown in the equation [107,109]

\[ D \propto r^{-3} (3 \cos^2 \theta - 1) , \] (1.13)

where \( r \) is the internuclear distance and \( \theta \) is the angle between the static field and the internuclear vector. For solution samples where isotropically tumbling molecules occur, \( D \) is averaged to zero [106]. In other words, the molecules move freely, and dipolar couplings are not observed. In contrast, when there are restricted motions, dipolar couplings are present, which enhance the broadening of the NMR resonance [106]. Line broadening is also due to sample heterogeneity like a liquid containing solid sample.
Magic Angle Spinning (MAS)

Magic angle spinning has been applied in order to correct the line shape and to improve the resolution of the solid state NMR spectrum. The line broadening from both dipolar couplings and susceptibility continuities can be corrected by quickly rotating the sample about the so-called magic angle $\theta = 54.7^\circ$ [107,109], so that the dipole-dipole term vanishes and the overall molecular motion becomes similar to the motion of the molecules in solution. This magic angle spinning (MAS) has been used in solid samples in order to average the chemical shift anisotropy [107,109]. In solution, dipolar splitting and chemical shift anisotropy are reduced to zero due to the rapid isotropic motion and the tumbling effects. As a result, the average orientation of the crystalline axes will be equivalent to the magic angle, which leads to sharp lines. Therefore, sharp lines at the isotropic chemical shift are obtained. Thus, this technique provides an enhancement of the signal-to-noise ratio and greater information content. The experimental setup of the sample probe with respect to the magnetic field is shown in Figure 1.6 where the sample is oriented with respect to the applied field at the magic angle.

![Diagram of Magic Angle Spinning](image)

**Figure 1.6** Magic angle spinning was performed by rotating at high speed. Solid sample inclined at the magic angle of $54.7^\circ$ to the static field.
X-ray Spectroscopy

X-rays are electromagnetic radiation, which are located between visible light and gamma rays in the electromagnetic spectrum [110-112]. X-rays are characterized by their relatively short wavelength ranging from 0.01 Å to 100 Å [110-112]. X-rays can be produced by converting the kinetic energy of charged particles into radiation resulting in a continuous spectrum. In comparison, monochromic x-rays can be obtained when exciting atoms collide with fast moving electron. These characteristic, monochromic lines are obtained when electrons from the higher shells descend to fill a hole in a lower shell. The energies of the characteristic lines depend on the energies of the two atomic shells involved (higher and lower shell). Electrons in the atoms are distributed in a succession of shells K, L, M, N, etc., which are labeled according to the decreasing energy, respectively. For each element, each shell has specific edge energy [113-115]. For example, K-edge energy corresponds to the energy, which is necessary to "knock” an electron out of the K shell.

The study in this dissertation was focused on XANES of calcium at the K edge. The objective was to monitor the absorption coefficient when an electron was ejected from the inner most shell of the atoms. This process produced information on the chemical environment of the calcium complex. In addition, x-ray absorption spectroscopy was used to investigate the chemical forms of calcium deposits, and to compare the calcium XANES spectra with the spectra of model compounds, such as hydroxyapatite and fatty acid calcium salts.
X-ray Absorption Spectroscopy

During the x-ray absorption experiment, x-rays are absorbed by an atom causing a core electron level from, K, L, or M shell to be ejected out of the atom into the continuum (Figure 1.7). The atom is in the excited state, and has an empty electronic core hole. Then, a higher-level core electron fills the core hole causing a fluorescence or Auger electron emission. Such processes occur in discrete quantities energy, which can be used to characterize the absorbing atom.

Figure 1.7 Description of x-ray absorption process

X-ray Absorption Near Edge Spectroscopy (XANES)

Biological specimens containing calcium have been studied using x-ray absorption near edge spectroscopy. The technique has been widely applied in the characterization of calcium in human skull bone, tissues, teeth, and milk. Peters et al. (2000) studied the structure of bone samples by the use of x-ray absorption spectroscopy, x-ray diffraction, and thermal analysis [117]. Kirtley et al. applied x-ray absorption spectroscopy to determine the nitrogen-chemical structures in DNA and related
molecules. [118,119]. The same authors assessed also the difference in the spectrum of the C=C bond in DNA from the bond in bovine serum albumin. Sugiura et al. reported Phosphorus K-edge x-ray absorption spectra of calcium phosphate compounds [120, 121]

**Figure 1.8** is a diagram of an x-ray absorption experiment. XANES requires a source of photon energy in the range of few keV. In the best cases, synchrotron radiation is used as a light source. XANES spectra were collected by the use of an x-ray beam emitted by the accelerated, relativistic particles in the storage ring traveling through a beam tube and slits into a monochromator. Two parallel crystals were used to send a single wavelength that is carried to an ion chamber that allows monitoring of the absorption of the samples. A second ion chamber located behind the sample measured the transmitted intensity. Detectors can be simple devices, like the gas ionization chamber in which detection of the ions is accomplished through the ionization of Ar, N₂, and CH₄. In addition, solid-state detectors such as, Ge solid state, scintillation counter, and photon diode can be used in fluorescence experiments [122].

![Figure 1.8 X-ray absorption experimental set up. Synchrotron radiation is used as an x-ray source.](image-url)
The energy dependence of the x-ray absorption coefficient, $\mu(E)$, at or above the absorption edge of a selected element, $\mu$, was measured. The absorption coefficient, $\mu$, depends on the x-ray beam intensity passing through the sample and the thickness of the specimen, as shown in the equation [116, 117]

$$\mu = (\rho Z^4/AE^3),$$

(1.14)

where $\rho$ is the density of the element of interest in the sample, $Z$ is the atomic number, $A$ is the atomic mass, and $E$ is the energy of the x-ray. $\mu(E)$ can be determined using the transmission or fluorescence mode.

In transmission mode, the absorption is measured based on the amount of light transmitted through the sample using the equations [116,117]

$$dI = -\mu I dx$$

(1.15)

$$\ln\left(\frac{I}{I_0}\right) = -\mu x$$

(1.16)

where $I_0$ and $I$ are the incident and the transmitted x-ray intensities and $x$ is the thickness of the sample.

In fluorescence mode, the filling of the deep core hole is detected through the determination of the x–ray absorption coefficient that is given by

$$\mu(E) \sim \frac{I}{I_0}$$

(1.17)

**X-ray Diffraction (XRD)**

X-ray diffraction (XRD) provides structural information from crystal or powder samples, in which the atomic constituents are periodically ordered [123,124]. X-ray
diffraction has been widely applied in the characterization of biomaterials [125-128]. Diffraction patterns are obtained for each sample due to the interference of the radiation with the crystal lattice or powdered samples. The diffraction pattern for any crystal depends on the distribution of the electron around the central atom, the thermal vibration of the central atom, the order of the atom in the unit cell, and the wavelength of incident monochromatic beam [123, 124].

Under ideal circumstances, all the diffraction patterns are obtained under the Bragg condition. The Bragg condition is met when the entire distance traveled by the x-rays in the crystal, 2a, is equal to nλ, as shown in the equation

\[ n\lambda = 2d \sin \theta, \]

where \( \theta \) is the angle of incidence of the x-ray beam with the crystal, d is the distance between the crystal plans, n is the diffraction order, and \( \lambda \) is the wavelength of the x-ray beam. Figure 1.9 is an illustration of an x-ray diffractometer. When coherent x-rays are scattered by an ordered lattice, the crystal structure can be determined. The x-ray tube has been commonly used as a source of monochromatic x-ray radiation of 8.4 keV.

![Figure 1.9 Schematic of an x-ray diffractometer.](image)
Chromatography

Chromatography is an analytical method that allows the separation of mixture of analytes as a result of their partitioning between the mobile phase and the stationary phase [129]. Tswett, a Russian botanist, first introduced the technique in 1906 [126]. His objective was to separate and isolate green and yellow chloroplast pigments. The separation was performed using a stationary phase of sucrose and a glass tube as a support column [130]. Since then, chromatography has continued to develop. The specific chromatographic techniques of thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and capillary electrochromatography (CEC) are discussed.

Thin Layer Chromatography (TLC)

TLC is a planar chromatographic method, which is used to identify or confirm the presence of biological markers in complex mixtures [133-136]. The stationary phase consists of a plate (glass, metal, or plastic) coated with a thin layer of a solid adsorbent (silica or alumina). Initially, a spot of the sample is placed at the bottom of this plate. Then, the plate is dipped into a pool of the mobile phase in a closed glass cylinder (Figure 1.10). The separation takes place when the mobile phase rises slowly with the sample via capillary force. As the solvent diffuses, the sample mixture interacts with the mobile phase and the stationary phase. The mobility of the components depends on the solubility in the mobile phase and the analyte adsorption properties. When the mobile phase reaches the top of the plate, the plate is removed from the glass chamber and dried. The separation of the components is visualized using ultra violet light or staining methods. The identification of analytes is based on the retardation factor ($R_f$) values.
The $R_f$ value of the compound is the ratio of the distance traveled by the mobile phase to the distance run by the analytes on the plate.

![Diagram of TLC system](image)

**Figure 1.10** A schematic of a TLC system: A.) The separation at $t_0$. B.) The separation over time.

**High Performance Liquid Chromatography (HPLC)**

In HPLC separation, the sample is injected into a packed column with an appropriate stationary phase [137-141]. **Figure 1.11** is a representation of the different components of a HPLC system. The separation mechanism is based on the partition of the analytes between the stationary phase and the mobile phase. The elution of the analytes is driven by pressure. The optimization of the separation is dependent on the stationary phase and the mobile phase composition [137-139]. HPLC experiments can be performed in either the isocratic mode or the gradient mode. In the isocratic mode, the solvent composition is maintained constant during the time of the experiment, whereas, in the gradient mode the solvent composition is varied throughout the run [137-139]. In both cases, the analytes are identified by migration times and through a peak spiking procedure.
The HPLC separation of most biological compounds has been performed using the reversed phase mode. In such cases, the stationary phase is composed of a non-polar matrix (C8, C18), and the mobile phase is a polar solvent. The mobile phase is usually a mixture of water and a polar organic modifier like methanol or acetonitrile [137-139]. In reversed phase-HPLC, separation is the result of hydrophobic interactions between the non-polar stationary phase and the hydrophobic group of the analytes. Hydrophilic analytes interact less with the stationary phase. Thus, they will elute faster. In contrast, hydrophobic analytes will elute at much longer time. Other types of stationary phases have been developed for specific compounds. For example, ion-pairing reagents have been bonded to the stationary phase in the separation of proteins [140-141].

Multiple detection techniques have been associated with HPLC. The most common is the ultra-violet (UV) detector. However, other detectors such as light
scattering, conductivity, Fourier transform infra-red (FTIR), laser induced fluorescent (LIF), mass spectrometry (MS), and nuclear magnetic resonance (NMR) have been used [142-146]. Among these combined techniques, HPLC-MS has become the most useful.

**Capillary Electrophoresis**

Capillary electrophoresis (CE) is an electrophoretic method where the analyte separation is based on the analyte in the presence of an applied electric field. A schematic of a CE instrument is shown in Figure 1.12. Both ends of the capillary are submerged in buffer vials where one end is the positive electrode, and the other end is the negative electrode. Then, a high voltage (max. 30 kV) is applied across the two electrodes. A small amount of sample at nanoliter level is injected at one end of the capillary and the sample migrates through the capillary. The injected species will diffuse depending on the electroosmotic flow, which is caused by the deprotonation of the free silanol group on the wall of the silica capillary. The positively charged counter-ions from the solution and the negatively charged ions at the capillary wall create a zeta potential, ζ, which causes the diffusion of the ions towards the cathode. The process enables the separation of the molecules regardless of their charge. The separation is optimized by controlling the analyte migration velocity. The migration velocity, μ, of the analyte depends on the electrophoretic mobility, μ_e, and electrosmotic mobility, μ_{eo}, yields [149-152]

\[ \mu = \mu_e + \mu_{eo}. \]  

(1.19)

Then, the migration time, t, of the analyte is given by

\[ t = \frac{l}{\mu E} = \frac{IL}{\mu V}, \]  

(1.20)
where $l$ is the length of the capillary to the detector, $L$ is the total capillary length, $V$ is the applied voltage, and $E$ is the electric field.

![Figure 1.12 A schematic of an CE system.](image)

The analytes in the capillary are detected using an UV or an LIF detector. The advantages of CE reside in its high separation power, its low sample size, and its short analyses time [153-156]. CE is a powerful analytical technique, which permits high selectivity separation of complex mixtures. In addition, CE can provide high sensitivity when coupled with mass spectrometry.

Other forms of CE have also been developed for the separation of more specific types of analytes (neutral analytes, proteins, lipids). Among those are capillary electrochromatography (CEC), micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis, and capillary isotacophoresis [157-159]. In this research,
CEC was used to characterize the cholesterol (esters) composition of the plaque. CEC provided high separation efficiency of charged and neutral analytes. In addition, high selectivity separation was obtained by the use of high concentrations of organic modifiers (methanol, acetonitrile, and tetrahydrofuran) [160]. In CEC, a column packed with silica particles (LC stationary phase) is used to perform the separation, as a result of the plug-like flow driven by the application of a voltage.

**Mass Spectrometry**

Mass spectrometry (MS) is used on structural elucidation of biomolecules [161]. When it is coupled with liquid chromatography (LC), the technique can provide molecular weight identification of biological compounds. Techniques, such as electrospray ionization mass spectrometry (ESI-MS), atmospheric pressure chemical ionization (APCI), and matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) have been used with success for structural information of organic and inorganic compounds [161-164].

**Electrospray Ionization Mass spectrometry (ESI-MS)**

In electrospray, the ionization process is obtained by use of an electric field. Charged droplets are formed at the exit of a capillary tube with a weak flux. The electric field induces the accumulation of charged droplets located at the end of the capillary. Then, the charged droplets are exposed to nitrogen, leading to the droplets shrinking due to evaporation. Finally, a quasi-molecular ion is released as a result of strong charge repulsions [161]. The ions that are formed from these processes are first selected by the mass analyzer, and then detected using an electron or photomultiplier detector. Figure 1.13 shows a scheme of an ESI-MS ionization mechanism.
Atmospheric Pressure Chemical Ionization (APCI-MS)

APCI-MS provides the ionization of labile and nonvolatile substances, so they can be measured in the mass spectrometer. The ionization of the analyte molecules results from collision with discharge from a corona needle containing ion gas. During the collisions, there is a loss of internal energy, which is accompanied by the interchange of a proton. Therefore, the process is known as gas phase ionization process. A consequence of the proton transfer is the ionization of the analytes. This occurs when the proton affinity of the analytes is greater than that of the solvent. Figure 1.14 is an illustration of the APCI-MS.

APCI-MS offers some advantages over conventional methods, such as electron impact ionization mass spectrometry [163]. The strength of the technique is that there is no need to introduce the liquid flow directly into the vacuum chamber.
Figure 1.14 Atmospheric pressure chemical ionization.

**Time-Of-Flight Mass Spectrometer (TOF-MS)**

In TOF-MS, ions of different mass are accelerated using a uniform kinetic energy. The ions with different velocities will reach the detector at different times for a given distance. The most common type of TOF-MS is the one that uses a pulsed ion source where a voltage pulse extracts the ions from the source. **Figure 1.15** is a design of a TOF-MS. A potential gradient between A and B of the field free flight tube allows the ions to travel with a uniform kinetic energy, and to be separated based on their m/z ratio at the repeller C. Finally the sorted ions are detected at D with photo-multiplier tube.

The main concern for this set-up is the resolution. However, the resolution can be optimized by increasing the ion flight time [164-166].
Scope of Dissertation

Atherosclerotic plaque development is a dynamic process in which plaque formation is in equilibrium with liquid blood and solid deposits. Although many studies have been performed, the process of this equilibrium with respect to the chemical composition of the deposits remains unclear. In addition, the factors that affect the equilibrium are not well understood. In order to better understand this equilibrium process, differences between bypass and coronary artery plaque deposits from the same heart were investigated. Bypass plaque deposits are chemical materials, which accumulate after bypass surgery. In contrast, native coronary artery deposits are associated with the materials that accumulate during the lifetime of the patient. Therefore, the chemical differences between the two plaques may imply changes in body metabolism in later life. Such changes may be related to factors, such as diet, lifestyle, and age. Previous studies conducted by Robinson and Murungi (1992) indicated that calcium and phosphorus concentration in bypass were an order of magnitude higher than in the native artery [10]. The complexities of the deposits depend on the acuity of the
disease. However, the effects of these components, and other factors that affect such equilibrium are not well understood. Studies in this dissertation were performed by the use of inductively coupled plasma mass spectrometry (ICP-MS), x-ray absorption near edge spectroscopy (XANES), nuclear magnetic resonance (NMR) spectroscopy, x-ray diffraction (XRD), and chromatography.

**Elemental Analysis By Use Of Inductively Coupled Plasma Mass Spectrometry**

The objective of these studies was to compare the distribution of the minerals in native and bypass plaque deposits and to investigate the chemical forms of calcium in both native and bypass plaque deposits. The ion metals (Ca, P, Mg, Zn, Cu, Cr, and Cr, F, and S, etc.) present in the plaque were characterized. To determine the chemical form of calcium in comparison with hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), the molar ratios of Ca to P were calculated for each sample. Then, this ratio was compared to the theoretical molar ratio of Ca to P in hydroxyapatite. The differences between calcified samples and the atheroma deposits were established by comparing the molar ratios of Ca to P.

**Solid-State NMR**

Magic angle spinning solid state NMR was performed on the plaque deposits from native and bypass coronary arteries, and on cholesterol, homocysteine, and homocystine model compounds. The method allowed the determination 1) of the chemical differences between the native and the bypass coronary plaque deposits and 2) the study of the interactions of calcium with homocysteine and cholesterol. In the $^{13}$C NMR spectrum of calcium-homocysteine, the chemical shifts of the carboxylate, alkene or carbon attached to the sulfur were examined. In addition, the contribution of the proteins in the plaque was
determined using the ratio of the carboxylic to the aliphatic peak areas in $^{13}$C NMR spectra.

**X-ray Spectroscopic Methods**

X-ray absorption, x-ray diffraction, and imaging (light microscopy, scanning electron microscopy and microtomography) techniques were used to examine the native and bypass coronary plaque deposits. These techniques were used to determine the distribution of the calcium within the plaque. In the early stage of the plaque formation calcium is distributed heterogeneously in the arterial walls. X-ray absorption methods were used to localize the calcium deposits and to investigate their chemical forms.

Ca K-edge XANE spectroscopy, light microscopy, and x-ray diffraction were performed on coronary plaque deposits, fatty acid calcium salts, and hydroxyapatite. In addition, silver staining methods were used to localize, and to identify the forms of calcium in the deposits. A comparison of the x-ray data of the deposits with model compounds allowed determination of the chemical forms of calcium in the calcified and atheroma plaques.

**Capillary Electrochromatographic (CEC) and High Performance Liquid Chromatography –Atmospheric Pressure Chemical Ionization Mass Spectrometric (HPLC-APCI) Analysis**

The lipids from the native and bypass coronary artery deposits from the same heart were extracted using the Folch method liquid extraction procedure. The chemical compositions were determined using HPLC, CEC, and TLC. In addition, APCI-MS was used to confirm the presence of cholesterol and its esters in chromatograms of the crude extracts.
References


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[131] Poole, C. F.; Poole, S.K. Anal chem. 1989, 61, 1257A-1266A.


CHAPTER 2
ELEMENTAL ANALYSIS OF PLAQUE DEPOSITS FROM HUMAN AORTA AND CORONARY ARTERIES

Introduction

Previous studies in our laboratory showed that calcium and phosphorus concentration in bypass arteries were significantly higher than in native arteries [1]. However, these samples were obtained from living heart bypasses that had complete obstructions. These deposits were obtained by drilling the clogged arteries. Due to the high risk of death, this medical operation is no longer practiced.

Minerals play a vital role in the proper function of the cardiovascular systems. Potassium, Na, Ca, and Mg play an important role in conduction and contraction of muscles including those of the heart. Studies using rats have shown that Cu deficiency results in degradation of the smooth muscle of the arteries and formation of coronary aneurysms [1,2]. A high Zn to Cu ratio results in hypercholesterolemia in a lot of animals [3,4]. Lead and Al have been implicated in some forms of heart diseases, especially hypertension. Aluminum has been known to interfere with the metabolism of Ca and Mg [3-5]. Aluminum may bind to phospholipids, which can cause the alteration of the lipid membrane of the arterial wall. Lead is known to have high affinity for sulfurhydryl groups, and can interfere with membrane function. Homocysteine has been associated with heart disease although its function is not known. The presence of homocysteine should result in an elevated sulfur concentration. Therefore, the sulfur concentrations as well as the concentrations of the other metals mentioned above were determined. The sulfur concentration can be used as an estimation of the presence of homocysteine and its analogs in the plaque deposits.
According to many studies, the elemental composition of the plaque deposits and the nature of the coronary plaque deposits are related to the age of the patients [8,9]. However, there is little information on the concentration of minerals in these different types of plaque. The objective of this study was to determine the concentration of numerous elements in various type plaques, and to note differences and similarities found in the results. Such results may be used to establish a correlation with disease, ages, gender, and lifestyle, which can be used as predictor of further events.

**Experimental Section**

**Sample Collection**

Dr. Richard E. Tracy of Louisiana State University Health Sciences Center in New Orleans, LA, provided the coronary arteries and the aorta samples. The plaque deposits were collected from the coronary arteries of unanimous cadavers for whom the cause of death was usually unknown. The plaque was removed carefully to avoid contamination from the intima. The deposits from each coronary artery were classified as soft or crystalline plaques. The classification was based on the visual examination. After their collection, the plaque deposits were placed in clean plastic vials, and stored in the freezer at –70 °C until analyzed.

**Sample Digestion**

An amount of plaque was weighed (~ 50 mg). It was placed in an acid cleaned beaker and 30 to 50 ml of concentrated nitric acid were added. The solution was heated at low temperature (~40 °C) on a hot plate for 48 hours. The volume was reduced to about 2 ml and the resulting solution was transferred quantitatively into a 10 ml volumetric flask and diluted with distilled water. Each sample was analyzed in duplicate. A sample blank was prepared by reducing the 50 ml of nitric acid to 2 ml through heating, and by performing the dilution to 10 ml with distilled water.
**Instrument**

The sample solutions were analyzed for metals using inductively coupled plasma (ICP) model (Optima 3000) online with a quadrupole mass analyzer. The limit of detection of the ICPMS methods of each element was given in Table 1.5.1 chapter I. The elemental composition of each sample was determined after the analysis of the standard solutions. Calibration curves were developed using standards with various concentrations of metals of interest (Ca, P, S, Mg, K, Na, Cu, Pb, and Cr) run under the same conditions as the samples. The calibration curves were used to calculate the concentration of metals in the plaque samples.

**Results and Discussion**

**Metal Concentrations**

The elemental compositions for the calcified and the atheroma plaques are shown in Tables 2.1 and 2.2. The description for each sample is shown in the corresponding legend. Samples 11, 12, 13, and 14 were taken from one heart, and the samples 22, 23, 24, and 25 were taken from another heart. It can be seen from these samples that there is a variation in metal concentration within the same heart or aorta sample.

In general, all the concentrations were at the mole/g level, except for Ca and P, which were at the mmole/g level in the calcified plaques. These two elements were about an order of magnitude higher in concentrations in the calcified plaque than in the atheroma plaque. The concentrations of sodium and magnesium were about an order of magnitude higher in the atheroma plaque than in the calcified plaque, albeit at significantly lower levels than the calcium and phosphorus concentrations. The copper and sulfur concentrations were marginally higher in atheroma plaque than in calcified plaque. However, no significant differences could be discerned for Zn, Fe, Al, Si, and Ba.
Table 2.1. Concentration of minerals in calcified plaques (µmoles/g)

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<thead>
<tr>
<th>Samples</th>
<th>Ca</th>
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<th>K</th>
<th>Mg</th>
<th>Zn</th>
<th>Cu</th>
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Table 2.2. Concentration of minerals in atheroma plaques (µmoles/g)

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<td>0.1</td>
<td>*</td>
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<td>0.3</td>
<td>*</td>
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</tr>
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<td>23</td>
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<td>30</td>
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<td>2.5</td>
<td>0.1</td>
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<td>*</td>
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<td>90</td>
<td>30</td>
<td>6</td>
<td>3</td>
<td>0.2</td>
<td>*</td>
<td>*</td>
<td>0.4</td>
<td>*</td>
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<td>8.0</td>
<td>95</td>
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<td>2</td>
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<td>0.1</td>
<td>*</td>
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Table 2.1-2.2a: Footnotes for 2.1 and 2.2

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Description</th>
<th>Age and Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Plaque from aorta</td>
<td>80 year old male</td>
</tr>
<tr>
<td>2</td>
<td>Plaque from an aorta</td>
<td>80 year old male</td>
</tr>
<tr>
<td>3</td>
<td>Plaque from an aorta</td>
<td>90 year old male</td>
</tr>
<tr>
<td>4</td>
<td>Plaque from an aorta</td>
<td>70 year old male</td>
</tr>
<tr>
<td>5</td>
<td>Plaque from a native coronary</td>
<td>70 year old male</td>
</tr>
<tr>
<td>6</td>
<td>Plaque from a native coronary</td>
<td>70 year old male</td>
</tr>
<tr>
<td>7</td>
<td>Plaque from a native aorta old</td>
<td>70 year old male</td>
</tr>
<tr>
<td>8</td>
<td>Plaque from a bypass coronary artery</td>
<td>70 year male</td>
</tr>
<tr>
<td>9</td>
<td>A mitral valve</td>
<td>70 year old white male</td>
</tr>
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<td>10</td>
<td>A mitral valve</td>
<td>70 year old white male</td>
</tr>
<tr>
<td>11</td>
<td>Plaque from aorta</td>
<td>55 year old male</td>
</tr>
<tr>
<td>12</td>
<td>Plaque from aorta</td>
<td>55 year old male</td>
</tr>
<tr>
<td>13</td>
<td>Plaque from aorta</td>
<td>55 year old male</td>
</tr>
<tr>
<td>14</td>
<td>Plaque from aorta</td>
<td>55 year old male</td>
</tr>
<tr>
<td>15</td>
<td>Plaque from an aorta</td>
<td>60 year old female</td>
</tr>
<tr>
<td>16</td>
<td>Plaque from an aorta</td>
<td>70 year old female</td>
</tr>
<tr>
<td>17</td>
<td>Plaque from an aorta</td>
<td>90 year old male</td>
</tr>
<tr>
<td>18</td>
<td>Plaque from thoracic aorta</td>
<td>40 year old female</td>
</tr>
<tr>
<td>19</td>
<td>Plaque from aorta</td>
<td>40 year old male</td>
</tr>
<tr>
<td>20</td>
<td>Plaque from abdominal aorta</td>
<td>40 year old woman with stroke</td>
</tr>
<tr>
<td>21</td>
<td>Lymphoma materials</td>
<td>40 year old woman</td>
</tr>
<tr>
<td>22</td>
<td>Plaque from an aorta with lesions</td>
<td>55 year old male</td>
</tr>
<tr>
<td>23</td>
<td>Plaque from an aorta with lesions</td>
<td>55 year old male</td>
</tr>
<tr>
<td>24</td>
<td>Plaque from an aorta with lesions</td>
<td>55 year old male</td>
</tr>
<tr>
<td>25</td>
<td>Plaque from an aorta with lesions</td>
<td>55 year old male</td>
</tr>
</tbody>
</table>

The mineral concentrations were higher in the calcified plaques than in the atheroma plaques. The calcium and phosphorus concentrations were at least an order of magnitude higher in calcified plaque than in atheroma plaque. The sodium and magnesium concentrations were about an order of magnitude higher in soft plaque than in crystalline plaque. Copper and sulfur levels were marginally higher in soft plaque than in calcified plaque. In some samples, the lead concentrations were quite high in the calcified plaque, but not in the atheroma plaque. The concentrations of the other metals (Zn, Fe, Al, Si, Ba) were
very low in concentration, and there were no discernable differences in their concentrations between calcified and atheroma plaques.

In these studies most of the plaque samples collected from women were atheroma plaque. However, the majority of samples from men hearts were crystalline. This may have either been accidental or a characteristic of deposits in the different sexes. The differences in composition between the physical form of atheroma and calcified plaques made the comparison of metal concentrations between samples from men and women impossible.

**Calcium to Phosphorus Ratio**

The molar concentrations of Ca and P in calcified and atheroma plaque are presented in Figures 2.1 and 2.2, respectively. The expected molar ratio of Ca and P in hydroxyapatite is shown as a line in the figures. In the calcified plaque, the Ca to P molar ratios are similar to that expected from hydroxyapatite (Figure 2.1). In the atheroma plaque, the P concentrations were significantly higher relative to Ca indicating the possible presence of phospholipids, phosphoprotein, adenosine triphosphate, guanine triphosphate, and phosphocreatine. (Figure 2.2). No conclusions could be drawn as to the chemical form of the Ca in this case.

The overall studies indicate a better correlation of calcium to phosphorus ratio in the hard plaque with hydroxyapatite than in the soft plaques. In some samples, the relative concentrations of phosphorus to calcium were too high to be hydroxyapatite. Thus, the phosphorus may be attached to organic molecules, and the calcium may complex with amino acid residues of protein, cholesterol, or phospholipids.
Figure 2.1 Calcium to phosphorus molar ratio in calcified plaque samples.

Figure 2.2 Calcium to phosphorus molar ratio in atheroma plaque samples.
### Table 2.3 Concentration of minerals in native and bypass coronary plaque deposits (µmole/g)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Ca</th>
<th>P</th>
<th>Na</th>
<th>K</th>
<th>Mg</th>
<th>Zn</th>
<th>Cu</th>
<th>Pb</th>
<th>Fe</th>
<th>Al</th>
<th>Si</th>
<th>S</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>180</td>
<td>220</td>
<td>109</td>
<td>3</td>
<td>22</td>
<td>0.6</td>
<td>0.04</td>
<td>0.03</td>
<td>0.2</td>
<td>1.1</td>
<td>0.6</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>71</td>
<td>160</td>
<td>9</td>
<td>29</td>
<td>0.2</td>
<td>0.6</td>
<td>0.004</td>
<td>0.01</td>
<td>1.0</td>
<td>0.4</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>85</td>
<td>80</td>
<td>26</td>
<td>16</td>
<td>0.4</td>
<td>0.02</td>
<td>0.007</td>
<td>1.0</td>
<td>2.0</td>
<td>0.2</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>750</td>
<td>763</td>
<td>171</td>
<td>61</td>
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<td>1</td>
<td>0.01</td>
<td>0.001</td>
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</tr>
<tr>
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<td>5000</td>
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<td>0.02</td>
<td>0.001</td>
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<td>1</td>
<td>1</td>
<td>80</td>
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<tr>
<td>6</td>
<td>85</td>
<td>270</td>
<td>293</td>
<td>12</td>
<td>13</td>
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<td>0.06</td>
<td>0.006</td>
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<td>6</td>
<td>0.5</td>
<td>140</td>
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<tr>
<td>7</td>
<td>1720</td>
<td>280</td>
<td>120</td>
<td>27</td>
<td>25</td>
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<td>0.06</td>
<td>0.006</td>
<td>1</td>
<td>5</td>
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<tr>
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Table 2.3a: Footnotes for 2.3

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<td>Native coronary artery</td>
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<td>2</td>
<td>Bypass coronary artery</td>
<td>55 year old male</td>
</tr>
<tr>
<td>3</td>
<td>Native coronary artery</td>
<td>70 year old male</td>
</tr>
<tr>
<td>4</td>
<td>Bypass coronary artery</td>
<td>70 year old male</td>
</tr>
<tr>
<td>5</td>
<td>Native coronary artery</td>
<td>47 year old male</td>
</tr>
<tr>
<td>6</td>
<td>Bypass coronary artery</td>
<td>47 year old male</td>
</tr>
<tr>
<td>7</td>
<td>Native coronary artery</td>
<td>56 year old male</td>
</tr>
<tr>
<td>8</td>
<td>Bypass coronary artery</td>
<td>56 year old male</td>
</tr>
<tr>
<td>9</td>
<td>Native coronary artery</td>
<td>55 year old male</td>
</tr>
<tr>
<td>10</td>
<td>Bypass coronary artery</td>
<td>55 year old male</td>
</tr>
</tbody>
</table>

The concentrations of the metals in the native and bypass coronary arteries were also determined using ICPMS. The concentrations of the minerals present in the bypass and the native plaque deposits are shown in Tables 2.3. The overall distribution of metals in the native and the bypass coronary artery plaque deposits is very different. Calcium and P were high in the native coronary plaque deposits, and much lower in the bypass coronary plaque deposits. The Ca to P molar ratio was higher than 1.66. Therefore, the calcium deposits in the native deposits are associated with hydroxyapatite. The low Ca to P molar ratio in the bypass is due to the variably high concentration of the P in the bypass deposits. These results might indicate that the deposits were not crystalline enough, and Ca was not in the hydroxyapatite form. In addition, essential elements, such as Mg, Zn, and S were found in the native and bypass deposits. The concentrations of Mg varied in the native and bypass deposits.

**Conclusion**

The concentrations of minerals were found to be higher in the calcified plaque samples than in the atheroama plaques. The differences in concentration of Cu and S were also observed in the calcified and atheroama plaques. It is not obvious whether or not the
high concentrations of Ca and P in the calcified plaques would affect the chemical form of calcium which was statistically estimated to be comparable to hydroxyapatite. In contrast, the above argument is rejected in the atheroma plaques where in average Ca and P concentrations were lower in the atheroma plaques.

From the hydroxyapatite Ca to P molar ratios, the minerals in the crystalline plaque primarily exist in the form of hydroxyapatite. However, the low mineral concentrations indicate that the major component of the calcified plaque is organic in nature. In comparison, hydroxyapatite is present in the atheroma plaque, but much of the phosphorus is organic in nature. The organic phosphorus may be an important. In contrast, calcium may play an equally important role at this and later stages. Finally, the low sulfur levels indicated that homocysteine was not a major component.

References


CHAPTER 3

CHARACTERIZATION OF NATIVE AND BYPASS CORONARY PLAQUE DEPOSITS BY USE OF SOLID STATE NMR

Part I. Investigation of the Chemical Differences Between Native and Bypass Coronary Artery Plaques from the Same Heart Using Cross Polarization Magic Angle Spinning Nuclear Magnetic Resonance (CP/MAS-NMR)

Introduction

The composition of atherosclerotic plaque has been studied using a combination of methods, such as scanning electron microscopy, x-ray spectroscopy, thermal analysis, and chemical analysis [1-7]. Weinmann et al. described the use of infrared (IR) spectroscopy to determine cholesterol and cholesterol esters present in the plaque deposits [8,9]. The liquid-crystalline phases of carotid plaque deposits have been determined by use of high-resolution $^{13}$C nuclear magnetic resonance (NMR) spectroscopy and $^{13}$C CP/MAS-NMR [10-12]. Warschawski et al. performed similar studies using a combination of MAS-NMR spectroscopy and two-dimensional Heteronuclear Overhauser Effect Spectroscopy (HOESY) on lipid membranes [13,14]. In this dissertation, CP/MAS NMR was used to investigate chemical differences between deposits from native and bypass coronary arteries from the same heart.

Experimental Section

Sample Preparation

Dr. Richard E. Tracy of the Department of Pathology of Louisiana State University Health Sciences Center in New Orleans provided the native and bypass coronary arteries. Both native and bypass coronary arteries were removed from the same heart of cadavers. The collected native coronary arteries were completely clogged.
whereas, the deposits in the bypass varied. After removal, the samples were kept frozen. For the NMR experiments, the arteries were opened longitudinally to expose the inside layers for the removal of the deposits. After collection, the samples were ground using a mortar pestle to facilitate the packing of the sample in the NMR rotor.

**Experimental Conditions**

The $^{13}\text{C}$ and $^1\text{H}$ CP/MAS spectra were acquired on a Chemagnetics Infinity 400 MHz (9.4 T) NMR spectrometer. The $^{13}\text{C}$ chemical shift standard was the methyl resonance of hexamethylbenzene ($\delta = 17.35$ ppm). The proton radiofrequency (RF) power, at the spectrometer frequency of 400.059499 MHz, was set to a 4.25 $\mu$s 90° pulse for cross polarization. The contact time was 100 $\mu$s. The number of scans was approximately 1000 and the delay between pulses was 3 seconds. Samples were spun in a 5 mm zirconium rotor with dry $\text{N}_2$ at a rate of a 5 kHz at room temperature. A spectral width of 50 kHz (20 $\mu$s dwell).

Phosphorus-31 spectra with proton decoupling were acquired at room temperature. The $^{31}\text{P}$ chemical shift was set with external 85% $\text{H}_3\text{PO}_4$ at $\delta = 0$ ppm. The phosphorus RF power was set equivalent to a 10 $\mu$s 90° pulse. Approximately 1000 scans were acquired with a delay between pulses of 1.5 seconds. A spectral width of 250 kHz (4 $\mu$s dwell).
Results and Discussion

Carbon-31 CP/MAS NMR

Using $^{13}$C solid state NMR, the native and bypass coronary plaque deposits were examined. Representative NMR spectra are shown in Figures 3.1 and 3.2. The $^{13}$C NMR spectra were complex in the region (0-75 ppm) corresponding to the aliphatic group of the steroid backbone of cholesterol and cholesteryl esters [15,16]. But, the complexity was not due to this. Rather it was doubtlessly due to the presence of hydrocarbon groups of the phospholipid, protein, and triglyceride contents of the plaque deposits.

<table>
<thead>
<tr>
<th>Chemical Shift (ppm)</th>
<th>Functional Group</th>
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</thead>
<tbody>
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<td>0-50</td>
<td>Aliphatic carbon</td>
</tr>
<tr>
<td>10-20</td>
<td>S-CH$_3$</td>
</tr>
<tr>
<td>110-135</td>
<td>Aromatic</td>
</tr>
<tr>
<td>55-65</td>
<td>$\alpha$C of the amino acid</td>
</tr>
<tr>
<td>105-130</td>
<td>Double bond</td>
</tr>
<tr>
<td>170-180</td>
<td>Carboxylate</td>
</tr>
</tbody>
</table>

The unsaturated carbons in cholesterol and cholesteryl esters have signals located between 120 to 130 ppm. The deposits from the native coronary arteries are usually firmer than those from the bypass arteries. Such physical changes between deposits may change the peak shape at 25 ppm. This signal was assigned to the chemical shift of
carbon of the methyl group in aliphatic side chain of cholesterol and cholesteryl esters, and may be restricted to motions due to the crystalline of the sample. Therefore, in the crystalline deposits, the signal of the methyl group, which is adjacent to the aliphatic group of cholesterol backbone, remains broad. Similar differences at ~55 ppm and 175 ppm resonance chemical shift were observed. The signal at 55 ppm was less enhanced in the deposit from the native artery than in the bypass, which could indicate a lower concentration of double bonds in the deposits from the native arteries than from the bypass arteries.

To examine the $^{13}$C NMR spectra of the plaques, the ratio of the carboxylate peak (~175 ppm) was compared to the total peak areas (0-75 ppm). The results in the different plaque samples are shown in Table 3.2. On average, the ratio was 1 to 5 suggesting that one carboxylate correlated with 5 other carbons. This 1 to 5 ratio does not correspond to a cholesteryl ester area ratio where the ratio is about 1 to 40 (Table 3.3).

Previous studies have shown that the cholesterol and cholesteryl esters composition constituted no more than 40% of these samples. The high carboxylic group content must originate in the remaining 60% of the plaque indicating a ratio of 1 to 3 or 1 to 4. This strongly indicates the presence of large quantities of protein in the plaque.

**Phosphorus-31 CP/MAS-NMR**

The $^{31}$P CP/MAS-NMR spectra of hydroxyapatite and phosphotidylcholine were used to identify the resonance chemical shifts of the phosphorous components in the plaques. The $^{31}$P CP/MAS-NMR spectra of the native and bypass coronary plaques obtained are shown in Figures 3.3 and 3.4. Two well resolved phosphorus peaks from organic phosphorus (-0.6 ppm) and hydroxyapatite (2.6 ppm) were identified in the
bypass plaque samples and all other soft plaque deposits. In contrast, only one $^{31}$P resonance peak was found in the crystalline plaque deposits, which corresponded to hydroxyapatite. Since atherosclerosis is a progressive dynamic process, calcification may also be a progressive dynamic process. However, it should be noted that the calcium and phosphorus concentrations are at the ppm level and cannot account for the overall crystallinity of the hard plaque. It follows that the bulk of this crystalline material is organic in nature. Furthermore, the crystalline material would be more physically stable than the soft amorphous plaque.

Figure 3.1 $^{13}$C CP/MAS-NMR of native and bypass coronary plaque deposits from the same heart.
Figure 3.2 $^{13}$C CP/MAS-NMR of native and bypass coronary plaque deposits from the same heart.
Table 3.2. Ratio of the peak area of the main functional groups present in the plaque deposits in comparison with model compounds

<table>
<thead>
<tr>
<th>Samples</th>
<th>Area Ratio Carboxylic/Aliphatic $^{13}$C NMR</th>
<th>Area Ratio Alkene/Aliphatic $^{13}$C NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart 1 Native</td>
<td>0.20</td>
<td>0.10</td>
</tr>
<tr>
<td>Heart 1 Bypass</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td>Heart 2 Native</td>
<td>0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>Heart 2 Bypass</td>
<td>0.20</td>
<td>0.15</td>
</tr>
<tr>
<td>Heart 3 Native</td>
<td>0.19</td>
<td>0.13</td>
</tr>
<tr>
<td>Heart 3 Bypass</td>
<td>0.10</td>
<td>0.05</td>
</tr>
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<td>Heart 4 Native</td>
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<td>Heart 4 Bypass</td>
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<td>0.14</td>
</tr>
<tr>
<td>Heart 5 Native</td>
<td>0.19</td>
<td>0.08</td>
</tr>
<tr>
<td>Heart 5 Bypass</td>
<td>0.18</td>
<td>0.07</td>
</tr>
</tbody>
</table>
### Table 3.3. Ratio of the peak area of the main functional groups present in the reference standards

<table>
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<tr>
<th>Standard Samples</th>
<th>Area Ratio Carboxylic/Aliphatic $^{13}$C NMR</th>
<th>Area Ratio Alkene/Aliphatic $^{13}$C NMR</th>
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</thead>
<tbody>
<tr>
<td>Cholesteryl linoleate</td>
<td>0.03</td>
<td>0.20</td>
</tr>
<tr>
<td>Cholesteryl oleate</td>
<td>0.03</td>
<td>0.10</td>
</tr>
<tr>
<td>Cholesteryl palmitate</td>
<td>0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>Fatty acid (hexadecanoic acid)</td>
<td>0.01</td>
<td>0.0</td>
</tr>
<tr>
<td>Tryglyceride (Propane 1, 2, 3 triyl tripalmitate)</td>
<td>0.06</td>
<td>0.0</td>
</tr>
<tr>
<td>Triglyceride (propane 1, 2, 3, triyl trimyrisate)</td>
<td>0.09</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Figure 3.3 $^{31}$P CP MAS NMR of native and bypass coronary plaque deposits.
Figure 3.4 $^{31}$P CP MAS NMR of native and bypass coronary plaque deposits from the same heart.

Conclusion

Magic angle spinning solid state NMR was successfully used to characterize the native and bypass plaque deposits from the same heart. Results indicate that the composition of the deposits in the native and bypass arteries are different in some
respects, but vary widely in composition. The large broad band on the $^{13}$C NMR at 175 ppm indicates the presence of significant quantities of protein in all deposits.

There appears to be more double bonds in the plaque in the bypass arteries than in the native arteries. The phosphorus in the native arteries seems to be primarily in the form of hydroxyapatite, while the phosphorus in the bypass and other soft tissue is likely due to organic-phosphorus compounds.

**Part II. Investigation of the Effects of Calcium and Homocysteine on Cholesterol by Use of Solid State $^{13}$C CP/MAS-NMR**

It has been shown that the presence of homocysteine in the blood is a flag for potential heart problems [17-20]. The function of homocysteine is not known, and the chemistry involved is not understood. Since homocysteine is an amino acid that has an easily oxidized thiol group, it can damage the endothelium cell by generating reactive free radical oxygen species. This process can promote clot formation, and homocysteine thiolactone can precipitate LDL. An increase of the interactions of LDL with endothelium cells leads to the formation of platelets aggregates [20]. Ca is present in coronary plaques and it is not known if homocysteine is present in the coronary plaque. However homocysteine is present in the blood at concentration ranging from 10 to 12 $\mu$mol/L. 80% of the homocysteine in the plasma is protein bound and the remaining 20% are oxidized homocysteine, homocysteine-cysteine and free homocysteine [21]. The questions that arise are the following (1) do homocysteine and Ca interact with or without cholesterol, and (2) what is the implication in the plaque formation?

To answer the above questions, the reactions between homocysteine, cholesterol, and Ca were studied chemically. These experiments were conducted using $^{13}$C NMR on
homocysteine, cholesterol, and the mixtures of homocysteine with Ca, cholesterol with Ca and homocysteine with cholesterol and Ca. The reactions were signified by changes in chemical shift or changes in the peak shape.

**Introduction**

The structures of homocysteine and its related sulfur containing amino acids are shown in Figure 3.5. These amino acids are highly reactive due to the thiol group, which can be readily oxidized [22-25]. In the past, most research was focused on the autoxidation properties of homocysteine. In comparison, few investigations examined the reactivity of homocysteine with metal complexes. Homocysteine damages the endothelium cells. This, in turn, leads to injuries on the arterial walls. The ability of Ca to form a stable complex with homocysteine has not been demonstrated yet. Recently, homocysteine has been associated with the risk of coronary diseases [25,26]. Future findings of the complexation of Ca with homocysteine and its analogs might help to explain the injuries and the healing process that take place in the coronary arteries.

Over the years, some evidence of the coordination of calcium with amino acid was reported [27,28]. These studies tried to gain a clear understanding of tissue calcification. Many research groups synthesized and characterized calcium amino acid complexes. Most results indicated that the complexes were either dimers or chain like polymers [27,28]. Furthermore, the coordination of amino acids with Ca through the carbonyl group shows that calcium is linked to oxygen [29,30]. For example Tang et al., performed potentiometric measurements, which explained that the oxygen in the carbonyl group was involved in the coordination of calcium based upon the slight depression of the $\text{Ca}^{2+}$ amino acid titration curve [22]. Similar work was also conducted by Maeda et al.,
using NMR spectra of $^{14}$N, $^{15}$N, and the $^{17}$O in the determination of the interactions of calcium with glycine, DL-alanine, and β-alanine. They found that binding of calcium to both amino acids was established by progressively adding Ca$^{2+}$ to an amino acid solution. While adding Ca$^{2+}$ ions progressively, the evidence that Ca$^{2+}$ was bound to both carboxylate groups within the complexes was determined based on further broadening of the NMR signals of the $^{14}$N and $^{17}$O nuclei [29-31]. Although no significant shift was observed, the authors concluded that Ca binds very weakly to the ligand. Using Raman and Infrared spectroscopies, similar observations were observed about calcium complexing with the ligands.

![Chemical structures of homocysteine and its analogs.](image)

**Figure 3.5 Chemical structures of homocysteine and its analogs.**
Experimental Section

Chemicals

Calcium chloride, homocysteine, and cysteine were purchased from Sigma-Aldrich (St-Louis, MO). Distilled water was obtained from the system provided by US Filter United State Filter Corporation.

Chemical Reaction

Calcium chloride (ca. 1.0 M), salt, and homocysteine (ca. 0.2 M) in aqueous solution at pH=7.4 were allowed to react in inert conditions for 24 hours. The precipitate was washed three times with distilled water and two times with pure ethanol. Then, the milky product was dried under a stream of nitrogen for 30 minutes. The final product was stored in a sealed vial in the refrigerator until further analysis.

Results and Discussion

\(^{13}\)C NMR Spectra of Calcium-Homocysteine

The \(^{13}\)C NMR spectra of cholesterol, Ca-cholesterol, homocysteine, Ca-homocysteine-cholesterol, and homocysteine-Ca complexes were reported. The \(^{13}\)C NMR spectra of free homocysteine and calcium-homocysteine are shown in Figure 3.6. Upon addition of calcium, \(^{13}\)C NMR spectrum of Ca-homocysteine shows the chemical shifts of the carboxylate at 176 ppm, and the secondary carbon at 56 ppm. The chemical shift ranging from 0 to 70 ppm indicates a major shift, in comparison with pure homocysteine. The downfield shift is indicative of calcium homocysteine interactions via the sulfur and the carboxylic group. There was no major carboxylic shift in the \(^{13}\)C Ca-homocysteine solid-state NMR spectrum. Perhaps Ca interacts weakly with homocysteine
at the carboxylic group. Possible rearrangement of the structural complex can also favor direct interactions of Ca with the sulfur group [22,23].

![Diagram of homocysteine and Ca-homocysteine complexes](image)

**Figure 3.6** $^{13}$C CP/MAS-NMR of homocysteine and Ca-homocysteine complexes.

The interactions of Ca with cholesterol with and without homocysteine were also studied. Both precipitates were analyzed using solid state $^{13}$C NMR. The spectra of the reaction products were compared with free cholesterol. The active sites of free cholesterol were previously described using solid state $^{13}$C NMR (Figures 3.7-3.9). In these spectra the chemical shifts of the carbon attached to the hydroxyl, the alkene, and the methyl groups were monitored. The Ca-cholesterol NMR spectra indicated a shift of the alkene. Hence, a change of the peak shape (120-130 ppm) was noticed. The results
illustrated that the alkene group interacts with Ca. The results imply that Ca may interact with cholesterol in the plaque. Such interactions might initiate phase changes of the plaque. In contrast, there were no major changes in the solid state $^{13}$C NMR of Ca-cholesterol-homocysteine compared to the solid state $^{13}$C NMR of free homocysteine. The active role of Ca might be inhibited by the presence of homocysteine.

Figure 3.7 Solid State $^{13}$C NMR spectra of Cholesterol/Ca precipitates.

These preliminary results are very promising. However, more studies needed to be performed in order to monitor the kinetics of the reaction of Ca-homocysteine-cholesterol. Due to the possible release of peroxide by homocysteine, the presence of oxidized cholesterol in the reaction products is expected.
Figure 3.8 Solid State $^{13}$C NMR of cholesterol/homocysteine precipitates.

Figure 3.9 Solid State $^{13}$C NMR of cholesterol/Ca/Homocysteine precipitates.
Effect of calcium and homocysteine on cholesterol stability: a proton NMR case study

Liquid $^1$H NMR was also performed on the reaction products. Slight chemical shifts of the proton attached to the alpha and the beta carbon were observed on the Ca-homocysteine solution NMR. The following cholesterol Figure 3.10 illustrates the proton active sites of the molecule. The changes of the chemical shift of these protons were monitored when Ca was added to the homocysteine solution. The changes of the chemical shifts were reported in Table 3.4.

Figure 3.10  Chemical structure of cholesterol. The bullets represent the proton active sites of the molecule.
Table 3.4 Effects of calcium and homocysteine on cholesterol conformation

<table>
<thead>
<tr>
<th>Active sites</th>
<th>Cholesterol</th>
<th>Cholesterol/Ca</th>
<th>Cholesterol/Hcy</th>
<th>Cholesterol/Ca/Homocysteine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1H NMR</td>
<td>1H NMR</td>
<td>1H NMR</td>
<td>1H NMR</td>
</tr>
<tr>
<td>A</td>
<td>5.35</td>
<td>5.40/5.70</td>
<td>5.40</td>
<td>5.35</td>
</tr>
<tr>
<td>B</td>
<td>3.50</td>
<td>3.50</td>
<td>3.60</td>
<td>3.40</td>
</tr>
<tr>
<td>R</td>
<td>0.68</td>
<td>0.68</td>
<td>0.67</td>
<td>0.69</td>
</tr>
</tbody>
</table>

The peaks in the $^1$H NMR spectrum of the cholesterol/Ca are broader than the peaks on the $^1$H NMR spectra of the cholesterol/homocysteine and cholesterol/Ca/homocysteine. This observation leads to the conclusion that the interactions of Ca with cholesterol are much stronger than in the cases of cholesterol/Ca/homocysteine.

Conclusion

The $^{13}$C and $^1$H NMR spectra of Ca-homocysteine, Ca-cholesterol-homocysteine, and Ca-cholesterol reaction products were compared with the NMR spectra of cholesterol and homocysteine. Observation of the spectrum of Ca-homocysteine suggests some differences in comparison with the free homocysteine NMR spectrum. These changes are mainly observed for chemical shifts ranging from 0 to 75 ppm. The presence of these extra resonance peaks is likely due to the formation of disulfite group, and their interactions with Ca. A major chemical shift change was not observed at the resonance chemical shift of the carboxylic group when calcium was added to the free homocysteine solution. However, the liquid $^1$H NMR of homocysteine and Ca-homocysteine revealed a slight proton chemical shift, as a result of adding Ca. This data shows that Ca might contribute to the formation of these aggregates, although strong interactions between Ca and the free homocysteine may not exist. The evidence of the formation of the disulfite
group may favor the presence of multiple carboxylic groups that increase the chance for
Ca to bind.

References


CHAPTER 4
CHARACTERIZATION OF NATIVE AND BYPASS HUMAN CORONARY ARTERY PLAQUE DEPOSITS BY USE OF X-RAY METHODS

Part I. High Resolution Three-Dimensional Visualization and Characterization of Coronary Atherosclerosis in Vitro by Synchrotron Radiation X-ray Microtomography and Highly Localized X-ray Diffraction

Introduction

Recently, a number of imaging techniques have been used to image atherosclerosis either in vivo or in vitro, including magnetic resonance imaging [1-4], angiography [5-9], ultrasonography [10], computed tomography (CT) [11], electron beam tomography (EBT) [12], magnetic resonance angiography [13], etc. The spatial resolution of most of these clinical imaging techniques is greater than 0.5 mm. Although they can identify and characterize complex atherosclerotic lesions, their resolutions are still not satisfactory for imaging the details of micron-sized plaque, such as coronary calcifications. High resolution images of atherosclerosis is needed for research purpose, particularly for calcification studies. Calcification is correlated very intimately with atherosclerosis [14, 15]. A close correlation between the extent of coronary artery calcification and the burden of coronary artery diseases (CAD) has been confirmed [10, 12]. Currently, EBT is the “gold” standard for calcium detection in the coronary arteries. Its in-plane resolution is approximately 0.5 mm, with a slice thickness of about 3 mm. To our knowledge, most EBT images are in two-dimensional (2-D). Images of coronary artery calcifications at higher resolution and in three-dimensional (3-D) have not been reported yet.
In this dissertation, high-resolution 3-D visualization of coronary atherosclerosis by use of synchrotron radiation x-ray microtomography (SR-XMT) method is reported. Like conventional CT, the SR-XMT technique measures volumetric information, and it provides pictorial views of the internal and external structure of materials. However, due to the low divergence of the x-ray beams produced by synchrotron radiation, x-ray microtomography can achieve approximately one thousand times better spatial resolution than that of conventional CT [16]. The in-plane pixel size in the present report was 13 µm and the slice thickness in the reconstructed 3-D images was 13 µm. In-situ x-ray diffraction (XRD) measurements were performed simultaneously by aperturing and localizing the x-ray beam onto the calcification in question. The approach of combining high-resolution 3-D visualization and in-situ XRD represents a significant advance in visualizing and identifying atherosclerotic calcifications.

**Experimental Section**

The monochromatic XMT measurements were conducted *in vitro* [17] on human atherosclerotic arteries that were removed from three white males. Their ages were 51, 55, and 70, denoted sample A (SA), sample B (SB), and sample C (SC), respectively [18]. From a series of radiograms taken at sample orientations in 0.25 degree increments, a tomogram was reconstructed in the interactive data language (IDL) programming environment (19) using a filtered back-projection algorithm [20]. The reconstructed slices provide the x-ray mass density at each point in a sample. The reconstruction was performed using a fast Fourier Transform algorithm following a re-gridding from a polar to Cartesian coordinate system [20]. Typical reconstructed volumes involved $658 \times 658$
Results and Discussion

The coronary arteries used in these experiments were completely or partially obstructed (Figures 4.1A and 4.1B). For the native arteries, only the lumen of the sample SA was open throughout the 6.8 mm long segment. The diameter of the lumen decreased monotonically from one end of the artery to the other by 57.5%. Although this artery was not completely occluded like the others, it still had a large amount of clustering on the arterial wall. Recent studies have shown that during plaque development, there is "remodeling" of the artery wall, which maintain the lumen size. As atherosclerosis progresses, the "remodeling" is overcome by plaque development, and stenosis occurs. [3, 13]. Therefore, visualization of arterial wall is more important than that of lumen for the characterization of the atherosclerotic disease.

Three types of plaque compositions were distinguished based on the brightness of the image. The low-density component was identified as lipid-rich deposits, while the medium density was fibrous plaque. The high mass density component was identified as calcified particles [10, 21]. These three phases were separately observed using the absorption profile (Figure 4.1C). The absorption of the fibrous plaque at 12.5 KeV x-ray energy was about 0.003 (twice as the fatty plaque). The calcium deposit had a higher absorption, i.e. approximately 10 times higher than the fibrous plaque. The cross section sequence images of the native arteries perpendicular to the z-, x-, and y-axis show the morphologies and the distributions of the plaque components in atherosclerotic arteries in
three different directions. Some calcified plaques were separately distributed as small clusters, while others were large continuous pieces along the longitudinal direction (z-axis). The calcification density was greater at the edge of the interior (Figure 4.1C). The bypass arteries showed less calcification in amount and size. The volume ratios of calcification to artery wall of the three natural samples were 3.7 ± 0.2%, 6.0 ± 1.3%, and 20 ± 3%, respectively. The ratios for the bypass specimens were only 0.025 ± 0.004%, 0.032 ± 0.007%, and 0.021 ± 0.002%, respectively [23]. These ratios were hundred times lower than in the native deposits.

The high-density plaques were confirmed to be calcium deposits by in situ powder x-ray diffraction. Figure 4.2 illustrates the x-ray diffraction patterns in the native and the bypass coronary arteries, respectively. The calcifications in native arteries were more crystalline than those in the bypass arteries. The equivalent 2θ scan diffraction patterns were obtained when the Debye-Scherrer rings were integrated (Figure 4.2C). Each ring or peak was indexed by diffraction features of hydroxyapatite (HAP), Ca_{10}(PO_{4})_{6}(OH)_{2}, with a hexagonal configuration with lattice constants of a = 9.424 Å, c = 6.879 Å. The fact that no single unknown peak was found indicates that the majority of the crystalline component of a calcified plaque was composed of HAP. In addition, there was an amorphous phase that contributed to the broadening of the peaks. Small HAP crystal deposits were observed on the arterial walls when scanning electron microscopy (SEM) was used [25-28]. The crystals were reported to be 1-30 µm in diameter, and they were heterogeneously distributed. The amorphous phase corresponds to the connective tissue that has a long-range periodic structure.
Figure 4.1 Cross sectional image sequences along the three different directions. Sample A, B, and C were from the coronary arteries of a 51, 55 and 70 year old white male, respectively. The presence of calcium is indicated by the color. Increasing concentration of calcium changes color from orange to yellow.
Figure 4.2. Powder x-ray diffraction patterns of calcium deposits. In situ XRD patterns were acquired at the same beamline (17) by narrowing the synchrotron radiation x-ray beam. The incident monochromatic x-ray energy was 25 KeV, equivalent to 0.496 Å wavelength that is 3 times shorter than that of the frequently used Cu Kα radiation. (A) Debye-Scherrer pattern of one calcium deposit in the SC native artery and (B) in the SB bypass artery.

In the translucent images of native and bypass arteries shown in Figures 4.3A and B, the morphology, the size, and the distribution of the calcified tissues can be visualized intuitively. A large amount of the calcifications in the native arteries are preferentially deposited along the longitudinal axes of the arteries, and they have cylinder like shapes (Figures 4.3C and D). Only a few small calcifications were found in the bypass arteries. The data suggest that larger calcium accumulations occurred over a longer period of time. It was also observed that not all large calcifications had closed cylinder-like shapes. On
the contrary, the calcifications confined by the artery wall had a curve-like shape (Figure 4.3E).

**Figure 4.3 (A-E). Visualization of calcification in coronary arteries.**

The x-ray absorption features of the calcium deposits in the native arteries are different from those in the bypass. The mass densities of the calcified plaques in native arteries are much higher at the edge than in the interior (Figure 4.4A), whereas the highest attenuations occur in the centers of calcium deposits in the bypass specimens (Figure 4.4B). This difference shows that the HAP crystals nucleated in the tissue matrix may develop or move towards the outer surface of the calcified tissue over time. This results in a more dense distribution or larger crystalline grains near the edges. The
attenuation coefficients, $\mu$, of the fatty and fibrous plaques are very close to those of the adipose and soft tissues (Table 4.1). Assuming the calcified plaque is only composed of HAP crystals (neglecting the trace amount of metals Mg, Na and Fe [28] and connective tissue, the attenuation contribution fraction, $x$, of HAP crystals in the edge or interior of the calcifications was estimated by use of the equation

$$\mu_{\text{measured}} = x \cdot \mu_{\text{HAP}} + (1-x) \cdot \mu_{\text{tissue}}$$

the results are listed in Table 4.2. The maximum attenuation coefficient is less than 1/3 of the value of pure HAP, and the resulting percentages of HAP range from ~4 to ~22%.

Figure 4.4 Experimentally measured linear attenuation coefficient of the calcified plaques. (A) In each native artery. The monochromatic energies are 13.5, 12.5 and 11 keV for the SA, SB and SC arteries, respectively. (B) In each bypass artery. The x-ray energies are 12.5, 12.5 and 15 KeV for the SA, SB and SC, respectively. The calcified tissues in the native arteries were selected from the planar cross-sections of 340, 460 and 320, respectively, and 56, 62 and 480 slices from the bypass arteries (Fig. 4.1A).
Table 4.1 Attenuation coefficients $\mu$ in cm$^{-1}$ for each component at various x-ray energies (29). The densities of HAP, soft tissue and adipose tissue are 3.14, 1.0 and 0.95 g/cm$^3$, respectively [29]. The values were taken from the website of National institute of standards and Technology.

<table>
<thead>
<tr>
<th>E (KeV)</th>
<th>$\mu_{\text{HAP}}$ (cm$^{-1}$)</th>
<th>$\mu_{\text{Tissue}}$ (cm$^{-1}$)</th>
<th>$\mu_{\text{Adipose-tissue}}$ (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>114.5</td>
<td>3.8</td>
<td>2.4</td>
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<td>12.5</td>
<td>76.7</td>
<td>2.6</td>
<td>1.7</td>
</tr>
<tr>
<td>13.5</td>
<td>59.8</td>
<td>2.1</td>
<td>1.4</td>
</tr>
<tr>
<td>15</td>
<td>45.2</td>
<td>1.6</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Table 4.2 Fitted contributions of HAP and soft tissue to the measured $\mu$ values in the calcified plaque.

<table>
<thead>
<tr>
<th>Contribution fraction (%)</th>
<th>At the $\mu_{\text{aver}}$ in native artery</th>
<th>At the $\mu_{\text{max}}$ in bypass artery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SA</td>
<td>SB</td>
</tr>
<tr>
<td>HAP</td>
<td>38</td>
<td>38</td>
</tr>
<tr>
<td>Tissue</td>
<td>62</td>
<td>62</td>
</tr>
</tbody>
</table>

Conclusion

SR-XMT is a very competitive technique for the high resolution and 3-D visualization of atherosclerosis.. Complementary techniques, such as SEM and XRD, provided a complete characterization of atherosclerotic plaques. Although SEM has higher spatial resolution than SR-XMT, it has a small 2-D viewing field.
Part II. Characterization of Calcium Deposits in Human Atherosclerotic Plaques Using X-ray, X-ray Diffraction, Light Microscopy, and Staining Methods

Introduction

Atherosclerotic plaque can be calcified or amorphous, according to the high or low calcium content, respectively. Although the terms calcified and atheroma plaques are used in the literature, other researchers refer to these as a fatty streak plaque or a plaque with lesion, respectively [30]. It should be noted that plaque deposits generally start with fatty streak, and they progress to form lesions. The different morphologies of the plaque motivated the use of several techniques for the study of plaque deposits. Different spectroscopic and imaging techniques have been used in order to gain more insight on the co-localization of fatty components and calcium in the plaque. Selective methods, such as tomography, magnetic resonance imaging, and histological studies have been performed to characterize calcification [31,32]. The use of high-resolution tomography method allowed distinction and quantification of the presence of calcium in clogged arteries [33]. Other methods include scanning electron and light microscopies, which were used to assess the structural features of the atherosclerotic plaques [34].

Recently, Sarig et al. have successfully demonstrated the association of calcium apatite and cholesterol in atherosclerotic plaques utilizing confocal microscopy [35]. In this study, the determination of the cholesterol distribution within calcified media was conducted using a fluorescence staining method. The fluorescence stain probe, filipin, has a high affinity for cholesterol and was able to show the location of cholesterol within hydroxyapatite. The study concluded that cholesterol was incorporated within the calcium-phosphate-cholesterol agglomerates. It should be noted that these methods focus
on the localization of the mineralized plaque. However, they cannot distinguish between the different possible chemical forms of calcium [34].

In order to distinguish between homogeneous, heterogeneous, and ulcerated carotid plaques, Fourier Transform Infrared, Fourier Transform-Raman, and Near Infrared spectroscopic studies have been performed [36,37]. Although such vibrational analyses can provide more information regarding structures and morphology of the plaque deposits, the results are usually complex due to possible interferences from the sample matrix. Another difficulty associated with these studies is the complexity and multiple steps required in sample preparations. Therefore, alternative methods have to be considered. Wang et al. developed a magic angle spinning nuclear magnetic resonance method, with minimum interference to characterize the different phases of human endarterectomy plaques [38]. Based on the NMR chemical shifts, the latter study concluded that the spectral properties of the phosphate groups in carotid plaques were similar to those of hydroxyapatite in bone. In earlier studies, chemical analysis prior to sample extraction, performed by Murungi and Robinson, highlighted substantial differences of calcium and phosphorus contents between “old” and “new” formed plaques. The terms “old” and “new” were used to describe the plaque deposits in native and bypass arteries, respectively [39].

The chemical form of the calcium during the progression of the plaque has not yet been thoroughly studied. For example, it is not clear whether hydroxyapatite is formed during the initial formation of the plaque or if it appears only in the progressive form. It is also not obvious that there are no other intermediates in the process of calcification.
Recently, x-ray synchrotron radiation spectroscopy has been applied in the investigation of the calcium forms in proteins [44]. Furthermore, studies on bone samples were performed using synchrotron x-ray absorption, x-ray diffraction, and thermal analysis. The results indicated that the sample’s mineral phase was mainly disordered calcium apatite with no evidence of calcium phosphate phase [44-46]. In fact, the latter studies cannot detect calcium phosphate, even if it were present in the sample. However, different bone samples showed different carbonate content. In addition, the size and the morphology of the bone mineral particles were not found to correlate with the type of the bone. In addition, X-ray absorption near-edge structure (XANES) was used to determine the complexes of different metal ions [41,42]. In particular, XANES was used to determine the configuration of Ca-binding sites in calmodulin (CaM) [46]. The XANES results were shown to depend on the ketone and carboxylic functional groups in Calmodulin.

In this study x-ray methods, such as XANES and x-ray diffraction were used to study human coronary artery plaque. In particular, the possible calcium forms in the plaque were examined. Different chemical forms of calcium may also exist in the native and bypass plaque deposits. Such differences might be due to the long life chemistry of the native coronary artery as compared to recent events in the bypass. Therefore, the methods used in this study provide a powerful tool for examining the coronary plaques from native and bypass coronary arteries.
Experimental Section

Materials

Calcium oxide (CaO), calcium hydroxide (Ca(OH)$_2$), and hydroxyapatite (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$) were purchased from Sigma Chemical Co. (St. Louis, MO). Stearic acid and palmitic acid calcium salts were obtained from Fluka (Milwaukee, WI). The coronary plaques were donated by the Department of Pathology, Louisiana State University (LSU) Health Science Center, New Orleans, LA.

Sample Preparation

Segments (20 to 40 mm in length) from the right half of the thoracic to abdominal aorta and the left circumflex of the coronary arteries were collected from cadavers, and they were subjected to XANES analysis without further pretreatment. The acquisition of the calcium XANES spectra was performed after exposing a segment of plaque wrapped with polypropylene (PP) film to the x-ray beam.

For the x-ray diffraction experiments, the heart plaque samples were dried under a stream of nitrogen gas for 24 hours. Then, the samples were ground into a fine powder using a mortar and pestle. The plaque samples that were removed from the left circumflex arteries could not be transformed into fine powder due to their amorphous nature. Thus, the x-ray diffraction analysis was limited to only crystalline samples.

Sample Processing for Light Microscopic Analysis

The coronary artery plaque deposit sections were processed differently depending on the experiments (SEM or light microscopy). Due to the complexity of the tissues, intensive sample preparation was required in order to obtain reliable results. Initially, the
tissue components were preserved by fixation to avoid composition change. For the purpose of electron microscopic techniques, the tissue was cut into thin slides. Utilizing tissue samples cut with different thicknesses, the depth profiling, and the mapping of Ca were performed using electron microscopy.

Cross section cuts of the coronary arteries were performed. The performance of the shape of the slides was examined before and after their fixation. Hence the results indicated that the slides were more regular and uniform when the samples were preserved prior to the cut. After that the samples were embedded in a supportive media [48]. The light microscopy process required 4% formaldehyde (formalin) and paraffin as an embedding medium. For scanning electron microscopy, the fixation process required 2% glutaraldehyde and epoxy as an embedding medium. After fixation, the microscope slides were treated with silver nitrate.

**XANES Spectroscopy**

All experiments were performed at the Center for Advanced Microstructures and Devices (CAMD) Synchrotron radiation source at LSU, Baton Rouge, LA. The XANES experiments were conducted using a beamline of x-ray light source operating at 1.3 GeV. X-ray beams are produced by the linear accelerator, and they travel through a slit before hitting the monochromator. The XANES spectra were recorded in total electron yield (TEY) mode at a double monochromator (DCM) beam line that was equipped with Si (111) crystals. The monochromatic crystal radiation in the region of interest was provided under the Bragg angle conditions. The intensity of the incident radiation was measured using ionization chambers, containing 100 mbar helium. Signals were detected
using a Lytle detector. The beamline was calibrated by the K-edge absorption that corresponds to the white line of CaO at 4043 eV. XANES spectra of synthetic compounds were recorded in transmission mode at room temperature (25 °C). The atherosclerotic plaques were recorded in fluorescence mode because of the limited amount of plaque samples. Winxas software was used for data analysis [49].

The XANES spectra were obtained by exposing the samples to monochromatic x-ray beams ranging from 3993 eV to 4093 eV. The absorption spectrum was monitored while scanning the monochromator. Initially, a smooth baseline was observed when the x-ray energy was increasing. This trend is continuous until an electron of the K shell is excited or ejected corresponding to an edge jump. The edge jump described the transition of an electron from the ground state to the target with a vacancy in the absorbing atom. The total absorption $\mu(k)$ above the absorption edge is given by [46]

$$\mu(k) = \mu_0(k)(1 + \chi(k)),$$

where $\mu_0(k)$ is the smoothly changing fraction of $\mu(k)$, which corresponds to the absorption coefficient of the isolated atom. The fine structure is given by $\chi(k)$, which are the effects of the interference between backscattered and outgoing photoelectron signal in the photo-absorption matrix element.

**X-ray Powder Diffraction**

X-ray powder diffraction data was obtained using a Bruker Advance D8 powder diffractometer equipped with a focusing Ge (111) primary monochromator (CuKα₁ radiation). Finely ground samples were placed on a zero background quartz sample
holder. Intensity data was collected at ambient temperature in the 2θ range, between 10° and 80°, with a step width of 0.02°, and a 2 s count time.

**Results and Discussion**

The Ca K edge x-ray absorption of calcium model compounds (Ca_{10}(PO_4)_6(OH)_2, fatty acid calcium salts, and calcium sulfide) was measured (**Figure 4.5**). The results showed that the spectrum of hydroxyapatite was characterized by one K edge peak, and several weaker broad peaks. Two points of inflection were present in the hydroxyapatite spectrum. The features described the presence of structural disorder due to the lack of crystalline form of the hydroxyapatite sample. The smooth edge of the spectra showed no inflection for the plaque samples. A less intense white line with first and second order inflections was observed in Ca K edge XANES spectrum of hydroxyapatite. Above the threshold, we observed shape resonances that resulted from multiple scattering in the calcium local environment. The energy difference between the lower points of the edge to the maximum point of the shape resonance was calculated, in order to compare the differences in the XANES spectra between the plaque samples and standard calcium salt. **Figure 4.6** illustrates the XANES spectra of the plaques and the model compounds.

![Figure 4.6](image)

**Figure 4.5 Ca K edge XANES spectra of model compounds.**
The amplitude ratio of the shape resonance to the edge jump was determined. There were no major differences of \( E \) (keV) between the plaque samples and the standard calcium salts, except for calcium sulfide, stearic acid, and levulinic acid calcium salts.

\[ \text{Figure 4.6 Ca K-edge XANES spectra of plaques and model compounds.} \]

Structural studies using x-ray absorption fine structures (EXAFS) showed a difference between the plaque deposits and calcium salt model compounds. The amplitude of the magnitude of the Fourier transform determined the radial distribution function of the neighboring atoms. In the hydroxyapatite spectrum the distance of Ca to
oxygen was given by the location of the first shell. A relative shift of the first coordination shell of the deposits to hydroxyapatite was observed. The amplitude and the frequency of the Fourier transform EXAFS oscillations depended on the neighboring atom, and the distance between the core atom and the ligand. Differences were observed in the higher coordination shell above 1Å in all the spectra. This phenomenon was an indication of possible differences in sample crystallinity. More crystalline samples tend to be more ordered (Figure 4.7).

![Figure 4.7 Ca K edge EXAFS of coronary plaque deposits and hydroxyapatite.](image)

The combination of XANES and EXAFS allowed us to observe both short and long-range order, i.e differences between amorphous and crystalline materials.
Additional data using x-ray diffraction of the plaque deposit samples may give more details about the crystallinity of the deposits.

**X-ray microanalysis**

Scanning electron microscopy on crystalline and atheroma (amorphous) deposits from native and bypass coronary plaques revealed irregular, rough, and amorphous surface features. Simultaneous characterization using electron probe x-ray microanalysis provides elemental composition of highly calcified and amorphous deposits. Qualitative differences were observed. Calcium and phosphorus have the most intense signal in both plaque segments (Figure 4.8). However, strong background interferences due to gold coating materials were observed.

![Crystalline deposits](image1)

*Figure 4.8 X-ray microprobe analysis of cross section cut of crystalline and atheroma deposits.*
X-ray Diffraction Results

X-ray powder diffraction analyses were performed on the crystalline samples from native and bypass coronary plaque deposits. Figure 4.9A-B showed the diffraction patterns of the crystalline plaques and hydroxyapatite. Ca hydroxyapatite was used to assign the x-ray diffraction patterns of the plaque deposits. The peaks on the x-ray diffraction of hydroxyapatite were accounted for the hexagonal space group P63/m, \( a = 9.426 \, \text{Å}, c = 6.865 \, \text{Å} \) (JCPDS # 861203).

Figure 4.9 A) X-ray diffraction of hydroxyapatite and plaque deposits from native and bypass coronary arteries of a 55 year old male. B) X-ray diffraction of hydroxyapatite and plaque deposits from native and bypass coronary arteries of an 70 years old male.
These results were in agreement with the published powder diffraction data [51]. However, the broader peaks found on the x-ray spectra of the plaques were an indication of the amorphous form of the plaques. Therefore, x-ray diffraction method can be used to differentiate crystalline plaques from amorphous plaques.

**Histochemical and Histopathological Studies of Native and Bypass Coronary Arteries**

The silver staining method was performed on microscope slides with plaque deposits and calcium salts (hydroxyapatite, calcium sulfide, and stearic acid calcium salts), in order to study the distribution and the chemical forms of calcium on the arterial walls. In this method the fixed slides were washed with silver nitrate reagent. Then, the silver nitrate reacted with calcium deposits to yield a dark brown spot [48,52,53].

A metal substitution method, known as Von Kossa’s technique, was performed to characterize the calcium deposits in the native and bypass coronary plaque deposits. In this method, calcium was replaced by silver and a metallic salt was formed with the anions of the calcium salt. The dark spots indicated the presence of the reduced silver. This revealed the location of the sites where calcium previously resided. The following reaction mechanism describes the Ca displacement by Ag. The effect of the Ca ligand in the results of the staining is not clear. Therefore, different Ca salts were also treated with the Von Kossa silver stain.

\[
\begin{align*}
\text{Ca}^{2+} \text{CO}_3^{2-} + 2[\text{Ag}^+ + \text{NO}_3^-] & \rightarrow \text{Ag}_2 \text{CO}_3 + \text{Ca}^{2+} 2\text{NO}_3^- \\
2\text{H}^+ + \text{Ag}_2 \text{CO}_3 & \xrightarrow{\text{Reduction by strong light}} 2\text{Ag} + \text{H}_2\text{O} + \text{CO}_3
\end{align*}
\]
The Von Kossa technique was applied to the detection of calcium deposits, and the determination of their forms in native and bypass plaque deposits (Figure 4.10). In Figure 4.11, the model compounds of Ca with silver stain are shown. The data indicated that the stained native coronary plaque deposits show dark spots that were larger than those in the stained bypass coronary plaque.

Figure 4.10 Microscope slides of native and bypass coronary arteries with no silver stains on the slides.

Figure 4.11 Stained calcium salts with silver nitrate.
Conclusion

The results indicated that the XANES Ca K edge spectra of plaque deposits followed similar trends. A slight shift of the Ca K edge spectra of the deposits relative to that of hydroxyapatite was observed. This shift might be due to a possible change in the Ca coordination. The EXAFS results demonstrated differences between the native and the bypass plaque deposits. In addition, the EXAFS Ca K edges exhibited differences in the spectra of the plaque and hydroxyapatite. The differences were demonstrated by a shift of the first coordination shell in both spectra, which might be due to a change in the Ca environment.

X-ray diffraction analysis indicated that only mineralized plaque deposits were able to provide diffraction patterns similar to hydroxyapatite. However, this latest method failed to provide any information from the soft plaque, in which the forms of calcium could be mainly organic. Furthermore, staining studies using Von Kossa method showed that the distribution of the calcium on the arterial walls was heterogeneous. Moreover, the staining method illustrated the formation of the large calcium aggregates in the crystalline deposits.

References and Notes


[21] The SR-XMT measurements were performed at the GeoSoilEnviroCARS (GSECARS) bending magnet beam line, sector 13 of the Advanced Photon Source (APS) at Argonne National Laboratory. We used various monochromatic energies of 11, 12, 12.5, 13.5 and 15 KeV on different samples to get best contrast images.

[22] Human atherosclerotic arteries were obtained from the Pathology Department of Medical Center at Louisiana State University in New Orleans. Each artery was cut to about 20 mm long and inserted longitudinally in a plastic straw. After packing separately each sample, each individual straw was sealed at the bottom with a holder that allows the sample to stand in the probe. The arteries were kept frozen with blue ice until performed XMT measurements.

[23] IDL 5.4: Interactive Data Language, Kodak / research Systems, Inc.


[26] Lumen diameter $D_L$ was determined by lumen area $S_L$, $D_L = 2(S_L/\pi)^{1/2}$. Artery wall thickness is $(S_A^{1/2} - S_L^{1/2})/\pi^{1/2}$, where $S_A$ is artery area. The areas were calculated using IDL programming tool based on the absorption difference between the artery and air. The area of sample holder was subtracted from the calculation to get a corrected result.

[27] The volume fraction of calcification was determined by thresholding the calcification’s and air’s absorption to distinguish calcium deposits and artery wall. The sample holder’s volume was deducted to correct the result.

[28] Integration was done using FIT2D program made by ESRF. Its web page is at http://www.esrf.fr/computing/expg/subgroups/data_analysis/FIT2D/.


CHAPTER 5
CHARACTERIZATION OF PLAQUE DEPOSITS USING CHROMATOGRAPHIC METHODS

Part I. Capillary Electrochromatography of Cholesterol and Its Esters

Introduction

Cholesterol is one of the main components of cell membranes and has several functions in the body, including the synthesis of certain hormones such as vitamin D and bile acid. However, too much cholesterol may be dangerous since it is believed that accumulation of cholesterol in the walls of arteries and veins accelerates the formation of atheroclerotic plaque and consequently inhibits blood transportation. In the human body, this accumulation and inhibition of blood flow can be fatal by causing strokes and heart attacks [1-3]. Thus, accurate analytical tools for characterizing cholesterol are needed.

Over the years, gas chromatography (GC) has been used to characterize cholesterol and its derivatives [4,5]. Usually, the GC analysis of a complex mixture of cholesterol derivatives provides poor resolution due to the structural similarity of these compounds. Therefore, capillary columns with high efficiency are necessary. Fused silica coated with 50% phenol and 50% dimethylsiloxane has been successfully tested to separate a mixture of eighteen cholesterol oxidation products [4]. However, baseline separation of all cholesterol derivatives were not obtained. In general, GC and GC/MS methods have serious limitations including the need for thermally stable columns and cholesterol derivatization [6-8].

Reversed phase HPLC with evaporative light scattering (ELS), refractive index, and electrochemical detection methods have also been reported for monitoring cholesterol derivatives [9-13]. Duncan et al.[11] performed the quantification of free, total, and esterified cholesterol extracted from human blood serum using HPLC. Suarna et al.[14] characterized
the oxidized lipid products extracted from human atherosclerotic plaques using reversed-phase HPLC, and quantified the $\alpha$-tocopherol and ascorbate present in the extracted materials. These authors [15] also separated and characterized extracted cholesteryl, oxo- and hydroxylinoleate from human heart. The identification of extracted materials was achieved by performing semi-preparative HPLC, and then individual fractions were collected and analyzed by use of mass spectrometry with chemical ionization. Murungi and Robinson [16] characterized cholesterol, cholesteryl arachidonate, palmitate, and stearate extracts from bypassed and non-bypassed heart plaque arteries using normal phase HPLC. In comparison with the existing electrophoretic separation techniques such as capillary electrophoresis (CE), HPLC has low column efficiency due to the need for pressure driven flow. In addition, there are limitations due to UV-cutoff of organic solvent, which is often close to the $\lambda_{\text{max}}$ of cholesterol and its ester derivatives. Although ELS or mass spectrometry detection could be employed to overcome this problem, the choice of mobile phase is limited and the solvent residues can still affect the signal-to noise ratio.

To minimize solvent waste and improve the efficiency of the column, capillary electroseparation methods have been performed to analyze neutral and charged species. Micellar capillary electrophoresis (MCE) using sodium dodecyl sulfate (SDS) is a versatile technique that separates both charged and neutral species. To separate very hydrophobic neutral analytes, such as cholesterol esters, this technique does not appear to be a useful complement to HPLC. This is because such analytes often bind strongly to the most commonly used micelles SDS and co-elute at the micelle migration time. To minimize the strong interactions between SDS micelles and the cholesteryl esters, addition of organic solvents to the MCE buffer is one option. However, the concentration of the organic modifier
that can be used is limited by stability of the micelle. The addition of urea to the MCE buffer may reduce the binding of hydrophobic molecules (e.g. corticosteroids) to SDS, although this methodology may mask detection of cholesteryl esters at low wavelengths. Nishi et al., use bile salts as another option for the separation of hydrophobic compounds [17]. However, experience in our laboratory indicates that the use of this surfactant results in significantly higher baseline noise at the low wavelengths which are required for detection of underivatized cholesterol. Furthermore, recently microemulsion electrokinetic chromatography (MEEKC) appears to be a promising technique for the separation of highly hydrophobic compounds [18]. However, selectivity of the technique appears to be limited due to band broadening. In contrast, polymeric surfactants or micelle polymers, when used as a pseudo-stationary phase show high tolerance to organic solvent with high separation efficiency [19-21].

Capillary electrochromatography (CEC) is a hybrid technique, which combines the high efficiency of CE with the high selectivity of HPLC [22,23]. The separation mechanism in CEC is guided by the electrophoretic mobility of the solutes and their partitioning between the stationary phase and the mobile phase. As a consequence, selectivity in CEC and HPLC is similar for neutral analytes. However, for charged species, it combines the attractive features of electrophoretic and partitioning effects [24]. The technique continues to grow rapidly as evident by more and more applications developed in the last few years [25-28]. Zare et al. separated 15 of the 16 polycyclic aromatic hydrocarbons (PAHs) using CEC with an isocratic mobile phase [25]. Recently, Yan et al.[26] separated all 16 PAHs using an ACN/H$_2$O gradient. Similarly, corticosteroids in biofluids have been analyzed in less than 14 minutes using gradient elution [27]. In addition, triglycerides and free and derivatized fatty acids in fish oil have been separated using CEC [28]. Therefore, CEC is well suited for the analyses of
very hydrophobic compounds. This is because unlike MCE, high levels (40-80% (v/v)) of organic solvents (acetonitrile, tetrahydrofuran and methanol) can be easily used in CEC to adjust selectivity.

In this study, CEC is used to separate cholesterol and its ester derivatives. Several chromatographic parameters such as buffer concentration, proportions of acetonitrile, tetrahydrofuran and water, voltage, and temperature were evaluated to optimize the CEC separation of a complex mixture of cholesterol derivatives with various chain lengths. The possibility of combining CEC with EKC (using a polymeric surfactant) for improved resolution of cholesterol esters is presented. To our knowledge, this is the first reported application of CEC for the analysis of cholesterol and its ester derivatives.

**Experimental Section**

**Reagents**

Cholesterol (5-cholesten-3β-ol), cholesteryl acetate (5-cholesten-3β-ol 3-acetate), cholesteryl n-butyrate (5-cholesten-3β-ol 3-butyrate), cholesteryl n-valerate (5-cholesten-3β-ol 3-pentanoate), cholesteryl n-hexanoate (5-cholesten-3β-ol 3-hexanoate), cholesteryl n-heptanoate (5-cholesten-3β-ol 3-heptanoate), cholesteryl n-octanoate (5-cholesten-3β-ol 3-octanoate), cholesteryl n-nonanoate (5-cholesten-3β-ol 3-nonanoate), cholesteryl n-decylate (5-cholesten-3β-ol 3-decanoate), cholesteryl palmitate (5-cholesten-3β-ol 3-palmitate), cholesteryl oleate (5-cholesten-3β-ol 3-oleate), cholesteryl linoleate (5-cholesten-3β-ol 3-linoleate), cholesteryl laurate (5-cholesten-3β-ol 3-dodecanoate) and Tris[hydroxymethyl]amino-methane (Tris) were purchased from Sigma (St Louis, MO). Tetrahydrofuran (THF) and acetonitrile (ACN) were obtained from Fisher (Springfield, NJ). Trifluoroacetic acid (TFA) and hydrochloric acid (HCl) was purchased from Mallinckrodt (Paris, KY).
Sample Preparation

Standards

Stock solutions of each analyte were prepared at concentrations of ~10 mg/mL in THF. A 300 µL aliquot of each analyte was mixed and the solution was diluted with 1000 µL of a mixture (50:50) of THF and ACN. The final concentration of each cholesteryl ester in a test mixture was ca. 0.60 mg/mL.

Human Atherosclerotic Plaque

Plaque samples were obtained from the Pathology department of Louisiana State University Medical School. The samples were collected from a 94-year old male cadaver. Because atherosclerotic plaques are potentially biohazardous, all glassware and cutting tools were carefully washed with a freshly prepared 10% Clorox solution. Lipid contents were extracted from 1.20 g of wet specimen using a mixture of hexane/methanol/water (10:2:2 v/v/v). The hexane phase was separated and the solvent was evaporated under nitrogen. The recovered dry lipid materials (Ca. 9.00 mg) were dissolved in 2 mL of mixture of THF and ACN for CEC analysis.

Buffer Preparation

Buffers of 5, 10, 15, 20, and 25 mM of Tris were prepared and the pH was adjusted to 8.0 using 10 % TFA. THF and ACN were added to the buffer and the final solution filtered using a polypropylene nylon filter with 0.45 µm pore size and sonicated for 10 min.

Instrumentation

Capillary electrochromatography (CEC) experiments were conducted using an HP 3DCE system (Hewlett-Parkard, Wilmington, DE). Data were collected using ChemStation software. The CEC columns (100 µm i.d. x 357 µm o.d. x 30 cm total length, 20 cm to the
detector) were packed with 3 µm Hypersil CEC C18 silica media according to the standard procedure described by Boughtflower et al.[29]. The electrochromatograms shown in Figure 5.2 were obtained using a commercial C18 packed column (100 µm i.d. x 357 µm o.d. x 30 cm total length, 20 cm to the detector) purchased from Unimicro Technologies, Inc. (Pleasanton, CA). The packed capillary column was preconditioned by applying the potential in 5 kV increments for 10 minutes up to 25 kV. In between injections, the CEC columns were conditioned for 5 min using 20 kV. All sample solutions were injected electrokinetically (10 kV, 3 sec.). All CEC separations were performed using 12-bar pressure applied to both ends of the column to prevent bubble formation. Unless stated otherwise (e.g., Figure 5.6), the capillary was kept at a constant temperature of 25 °C. The elution of the analytes was recorded using a photodiode array detection system operated at three different wavelengths (200, 214, and 220 nm) with % RSD of approximately 5.00 for the migration time of cholesterol and its ester derivatives. Since the best signal-to-noise ratio was observed at 200 nm, this wavelength was used throughout the remainder of this study.

Results and Discussion

Cholesterol is a fat-soluble molecule. It belongs to a class of biological compounds called sterols. Like all alcohols, it forms esters with fatty acids. Thus, the ester derivatives of cholesterol are obtained by replacing the proton of the OH group by a fatty acid backbone with a different chain length. Figure 5.1 shows the chemical structures of cholesterol and the cholesteryl ester derivatives used in this study. The influence of operating parameters such as type of acidic modifiers, organic solvent composition, buffer electrolyte concentration, temperature, and applied voltage were studied to optimize the CEC separation of these cholesterol esters.
Figure 5.1 Chemical structures of cholesterol and twelve ester derivatives.

Effects of Organic Modifiers.

A stock solution of 25 mM Tris at pH 10 was prepared. To avoid the risk of mixed retention and the dissolution of the silica bed, HCl and TFA were tested as acid modifiers to adjust the pH of the running buffer to 8.0. Figure 5.2 compares the electropherograms for the separation of cholesterol derivative mixture using isocratic CEC with HCl and TFA as acid.
modifiers. Addition of HCl resulted in peak splitting and failed to prevent unwanted interaction between the stationary phase and the analytes (Figure 5.2A).

![Figure 5.2 Electropherograms of a mixture of cholesterol and twelve ester derivatives at pH 8.0. Using 25 % (v/v) THF/ 70 % (v/v) ACN /25 mM Tris. The pH of the mobile phase was adjusted before the addition of the organic modifiers with (a) HCl and (b) TFA. Conditions: 20 cm x 100 µm i.d. fused silica capillary packed with 3 µm ODS particles; 200 nm UV detection; 25 kV voltage, electrokinetic injection (10 kV for 3 sec). The peak identification is listed in Figure 5.1.](image)

In contrast, when the pH of the Tris was adjusted with TFA, better peak shapes (absence of peak splitting) were observed (Figure 5.2B). The background current was 23 µA and 14 µA using HCl and TFA, respectively. It is possible that the peak splitting observed with the use of HCl may be due to joule heating cause by higher background current. To improve peak shape
and resolution by suppressing interaction of the solutes with the silanol groups, all other CEC separation were performed with Tris buffers adjusted to pH 8.0 with TFA. Cholesterol palmitate (11) and cholesterol oleate (12) did not elute even after 65 minutes. Further optimization was performed to reduce the analysis time.

**Optimization of Mobile Phase Composition**

The effects of mobile phase composition were also investigated. Solvent strength and solvent UV wavelength cutoff were considered in the separation of cholesterol and its cholesteryl esters. It is well known that THF has higher elutropic strength than most solvents. It was found to be a good solvent candidate for fast, high efficiency separation of cholesterol derivatives. In contrast, the UV cutoff for pure THF is around 214 nm. This would likely increase the noise level and decrease the linear working range. However, the addition of H₂O and ACN decreases the relative background absorbance due to the dilution of pure THF. In addition, the background effects of UV-absorbing solvents appear to be less critical in CE than in HPLC, probably because of the use of the shorter pathlength in CE [30]. Consequently, a series of experiments was conducted to optimize the CEC of 13 components of cholesterol derivatives using various volume fractions of THF and ACN at a constant pH 8.0, and buffer composition of 25 mM Tris. **Figure 5.3** shows the separation profiles of cholesterol and its ester derivatives using various percentages of THF and ACN. Although cholesterol (peak 1) is less hydrophobic than the ester derivatives, it is more retained than the analytes 2, 3, 4, 5, and 6 (**Figure 5.3**). This suggests that cholesterol retention is likely governed by hydrogen binding interactions between the hydroxyl groups and the free silanols on the wall of the column. Therefore, cholesterol elutes as a result of polar and reversed-phase electrochromatographic interaction. In contrast, the neutral ester derivatives of cholesterol elute from less to the more
hydrophobic. Thus, the esters are separated based primarily on differential partitioning into the alkyl-bonded phase. For example, cholesteryl oleate (12) and cholesteryl linoleate (13) each have 18 carbons on the ester side chain. However, cholesteryl linoleate (13) is more hydrophilic (due to the presence of an extra double bond) and elutes before cholesteryl oleate (12). As expected, migration time is reduced as a result of increasing THF concentration. Overall, the elution order is not solvent dependent.

Figure 5.3 Effect of mobile phase composition on the CEC separation of cholesterol derivatives. Conditions same as Figure 5.2 except TFA was used for pH adjustment, and mobile phase (THF/ACN) composition was varied.
When the volume ratio of THF was increased from 25% to 55% (v/v), the electroosmotic flow increased by 10%. It is clear that an increase in the solvent strength (i.e. increasing THF concentration) decreases the total separation time at the expense of lower resolution and k’ values. At 25% (v/v) THF/70 % (v/v) ACN, the total separation is very long and the last four cholesteryl derivatives (10, 11, 12, and 13) did not elute even after 80 minutes. Increasing the THF concentration to 35% (v/v) gives an elution window of about 70 minutes from the electroosmotic flow marker to the elution of cholesteryl laurate (peak 11). All of the analytes elute in 20 minutes using 55% (v/v) THF. However, cholesterol and the twelve-ester derivatives in the mixture are not well resolved. Taking into consideration the resolution and speed of analysis, 35% (v/v) THF was used to further optimize separation conditions for the mixture of cholesteryl esters.

**Effect of Tris Concentration**

The effect of Tris concentration on the CEC separation of cholesterol and its ester derivatives was studied using 5 mM, 20, and 25 mM of Tris buffered at pH = 8.0. As shown in Figure 5.4, both retention time and resolution of the analytes increase with increasing Tris concentration from 5-25 mM. The increase in migration time is due to the interactions of Tris with the silica surface, which can alter the zeta potential, and thus reduce the electrosmotic mobility from $12.24 \times 10^{-5}$ to $6.29 \times 10^{-5}$ cm$^2$ V$^{-1}$ sec$^{-1}$. In general, resolution for most analytes increases with increasing Tris concentration due primarily to improved stacking during electrokinetic injection. The use of even higher Tris concentration (>25 mM) was not feasible due to longer retention times, which in turn leads to band broadening. Thus, a mobile phase of 25 mM Tris was chosen in all subsequent experiments.
Figure 5.4 Effect of Tris concentration on the CEC separation of a mixture of cholesterol and twelve ester derivatives. Conditions same as Figure 5.2 except TFA was used for pH adjustment, Tris buffer concentration was varied, with 35 % (v/v) THF/ 60 % (v/v) ACN.

Effect of Applied Voltage

The influence of the applied voltage on the efficiency, resolution, and the analysis time of the cholesterol esters were next evaluated using a mobile phase of 25 mM Tris in 35% (v/v) THF/60% (v/v) ACN/ 5 % (v/v) H₂O at 25 °C. As anticipated, a higher voltage reduced the retention times due to an increase in the net velocity from 27.2 mm/s at 25 kV to 52.5 mm/s at 30 kV. At 30 kV, the short chain esters and cholesterol elute faster with poor resolution. In contrast at 15 kV, separation of the mixture takes longer with less efficient peak separation. Based on these results, the best resolution with higher efficiency was obtained using 25 kV (see Figure 5.5).
Figure 5.5 Effect of applied voltage on the CEC separation of a mixture of cholesterol and twelve ester derivatives using 30, 25 and 15 kV. Conditions same as Figure 5.2 except applied voltage was varied from 15 kV to 30 kV. Mobile phase composition was 25 mM Tris, 35 % (v/v) THF/60 % (v/v) ACN/ 5% (v/v) H₂O, (pH adjusted with TFA to 8.0).

Temperature Studies

An increase in temperature from of 20 to 60 °C was investigated in the CEC separation of cholesterol derivatives. As shown in Figure 5.6, retention continues to decrease with an increase in temperature, whereas resolution between adjacent peaks first improves than deteriorates. An increase in temperature from 20 to 60 °C at 25 kV resulted in a 50% reduction in analysis time due to an increase in electroosmotic velocity caused by lower electrolyte viscosity. The peak resolutions significantly deteriorated at temperature > 40 °C.
Based on these results, a temperature greater than 40 °C was chosen as optimum since it produced baseline resolution of cholesterol and its ester derivatives in less than 40 minutes.

Figure 5.6 Effect of temperature on the separation of a mixture of cholesterol and twelve ester derivatives. Conditions same as in Figure 5.6 except 30 kV was used and the temperature was varied from 20 °C to 60 °C.

Effect of Pseudo-Stationary Phase: Polymeric Sodium N-Undecanoyl-Glycinate

It is now well documented that polymeric surfactants are stable even at high concentrations of organic solvent, whereas normal (unpolymerized) surfactants are not [17-19]. To investigate the effect of the polymeric surfactant, poly-sodium N-undecanoyl-L-glycinate (poly SUG), separation voltage (30 kV) and temperature (25 °C) were chosen for which the chromatographic resolution was relatively poor. Electropherograms using 25 mM Tris buffer with and without 5 mM of poly SUG at 30 kV are shown in Figure 5.7a and 5.7b, respectively. With no polymeric surfactant, the analytes are more retained because of
the hydrophobic interaction between the analytes and the hydrophobic chain of the stationary phase. The addition of (poly SUG) affects migration time and peak resolution. Shorter migration time and improved resolution were observed due to interactions between the polymer and the stationary phase, which in turn reduces the hydrophobic interactions of the analytes with the stationary phase. In addition, critical peak pairs (peaks 3 and 4; peaks 1 and 7) were baseline resolved. Therefore, due to improve efficiency upon the addition of polymeric surfactant, the overall analysis time was reduced from 40 min to 35 min. Further studies are underway to determine why only efficiency and not selectivity was influenced on the addition of polymeric surfactant.

![Figure 5.7](image-url)

**Figure 5.7** Effect of polymeric sodium N-undecanoyl-L-glycinate on the CEC separation of a mixture of cholesterol and twelve ester derivatives. Conditions same as Figure 5.6 except separation was performed at 30 kV and 25 °C.  

a) Tris buffer (25 mM, pH 8)/THF/ACN (5:35:60 v/v).  

b) Tris buffer (25 mM, pH 8)/THF/ACN (5:35:60 v/v); with 5 mM polymeric sodium N-undecanoyl-L-glycinate.
CEC Separation of Crude Extracts From Human Atherosclerotic Plaques

The samples involved in these CEC studies were mature plaques. The origin of such plaques results from progressive alteration in the structures of the arterial walls [31]. Studies have revealed that in later years, atherosclerotic plaques contain complex lipid deposits, which are essentially large quantities of cholesterol and cholesterol esters. To identify the lipid deposits, lipids extracted from plaque were removed from a segment of a human aorta and injected into the CEC capillary. Figure 5.8 shows the presence of the cholesterol, cholesterol linoleate, and cholesterol oleate contents in the plaque samples. As calculated based on peak area, the lipid extracts from the plaque sample contains 55% of cholesterol deposits with 20% cholesterol linoleate (13) and 24% cholesterol oleate (11).

Figure 5.8 CEC separation of crude extracts from atherosclerotic plaque of a human aorta. Conditions are: Tris buffer (25 mM, pH 8) /THF/ ACN (5:35:60 v/v); with 5 mM polymeric sodium N-undecanoyl-L-glycinate using a 25 cm x 100 µm i.d. fused silica capillary packed with 3 µm ODS particles; 200 nm UV detection; 30 kV voltage, electrokinetic injection (15 kV for 6 sec).
Conclusion

This work has fully demonstrated that CEC could be used to characterize cholesterol and cholesteryl esters extracted from atherosclerotic plaque deposits. In the CEC separation the peak shapes of cholesterol derivatives were improved considerably when TFA was used as an additive to adjust the pH of the running buffer to eight. Based on the poor solubility of the cholesterol esters, a running buffer with a high concentration of organic solvent (THF and ACN) was used as the mobile phase. The use of the polymeric surfactant, in the CEC buffer decreased analysis time and improved resolution between two critical pairs of ester derivatives (3/4 and 1/7). Thus, baseline resolution of the 13-component mixture of cholesterol was achieved with a separation time of less than 40 minutes with no need of prior derivatization. Alternatively, in the absence of the polymeric surfactant, an increase in temperature to 40 °C provided baseline separation of the 13-component mixture on a similar time scale. The optimized CEC method in the presence of the polymeric surfactant allows the characterization of cholesterol, cholesterol linoleate, and cholesterol oleate present in human aorta plaque.

Part II. Determination of Cholesterol and Its Esters in Plaque From Bypass and Native Arteries From the Same Human Hearts Using Liquid Chromatography/ Atmospheric Pressure Chemical Ionization Mass Spectrometry

Introduction

Many analytical methods have been used to study the composition of plaque deposits including gas chromatography coupled with mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) to monitor the levels of cholesterol and related compounds in plasma, tissues and food [32-35]. HPLC methods have often been utilized as an alternative approach for the characterization of cholesterol and its ester derivatives [32-36]. Detection
methods, such as light scattering, refractive index, or ultra visible light have also been applied [37-40]. In each of these cases, the identification of the targeted analytes was determined using migration time and spiking methods. In addition, mass spectrometry methods have been used to identify the myriad of compounds involved [40-47].

Earlier studies in our laboratory showed that the calcium and phosphorus concentrations in bypass deposits were significantly higher than in native artery deposits [16]. However, it should be noted that these samples were obtained from bypass arteries that had become completely occluded. In contrast, current samples were obtained from cadavers and were often newly formed and different in texture.

The objective of this research is to examine the cholesterol components of deposits in native and bypass deposits from the same heart and to note any differences. Such results can be used to establish correlation between the cholesterol profile and the risk factors associated with atherosclerosis. Therefore, quantitative determinations of cholesterol and cholesterol esters were performed by use of chromatography and mass spectrometry. Attempts to use liquid chromatography coupled with electrospray mass spectrometry were unsuccessful because of the complexity of the spectra. Atmospheric pressure chemical ionization mass spectrometry was therefore used as the detection system since it avoids the necessity of derivatization. This technique was successfully applied in this study. Large quantities of other compounds were shown to be present. These have been qualitatively studied using $^{13}$C and $^{31}$P solid state NMR [49].

Due to the complexity of the mixture of the crude extracts, UV and APCI detection techniques were used to identify cholesterol and its esters in the plaque deposits. Successful use of this method allowed a full characterization of cholesterol and its esters in the plaque deposits.
Experimental Section

Reagents and Chemicals

The standards of cholesterol, cholesteryl linoleate, cholesteryl oleate, cholesteryl palmitate, and stearate were purchased from Sigma (Milwaukee, WI) (Figure 5.9). Isopropanol, acetonitrile, and formic acid were of HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA). The human coronary plaque deposits were obtained through collaboration with Dr. Richard E. Tracey from the department of Pathology of Louisiana State Health Centers in New Orleans, LA.

Figure 5.9. Structures of cholesterol and its esters.
Sample Preparation

Standards

Standard solutions of cholesterol, cholesteryl arachidonate, cholesteryl linoleate, cholesteryl oleate, and cholesteryl palmitate were prepared at concentrations of approximately 1.0 mg/mL in a 50:50 mixture of isopropanol and acetonitrile.

Atherosclerotic Plaque

The plaque samples from native and bypass coronary arteries were collected from cadavers as listed in Table 5.1. The plaque deposits were obtained from the inner lining of an artery. The sampling of the plaque was achieved by opening the arteries longitudinally and peeling off the calcified and the fatty materials located in the interior of the tunica intima [31, 50]. This study was to ascertain any differences in composition in bypass plaque (new) and native plaque (old). Hence, the results may signify a change in body metabolism.

Plaque Extraction.

Approximately 0.1 – 0.2 g of samples were weighed. The cholesterol and its ester derivatives were extracted with a mixture of chloroform/methanol/water (10:2:2 v/v/v) [51]. The chloroform phase was separated, and the solvent evaporated under nitrogen. The recovered dry lipid materials from the plaques (9.0 -5.0 mg) were dissolved in a 500 µL mixture (50:50) of isopropanol/ acetonitrile for LC/ APCI MS analysis.

Instrumentation

HPLC APCI-MS System.

The cholesterol and cholesteryl esters in the crude extracts from the plaque deposits were examined on a Mariner™ API-TOF Workstation from Applied Biosystems (Framingham, MA)
coupled to an HP 1100 HPLC system with an autosampler and an UV-VIS detector (Paolo Alto, CA). The separation was achieved using a Bischoff ProntoSil C$_{18}$ column, 120 Å, 3 µm, 100 x 4.6 mm (Leonberg, Ger) with a solvent flow rate of 1 mL/min. A mixture of 30% isopropanol, 70% acetonitrile, and 0.1% formic acid was used as the optimum eluent for separation of the mixture of cholesterol and cholesteryl ester derivatives considering a 10 µL injection size.

<table>
<thead>
<tr>
<th>Plaque deposit</th>
<th>Age</th>
<th>Gender</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart 1 Sample 1</td>
<td>70</td>
<td>Male</td>
<td>Native coronary artery</td>
</tr>
<tr>
<td>Heart 1 Sample 2</td>
<td>70</td>
<td>Male</td>
<td>Bypass coronary artery tied to the left circumflex</td>
</tr>
<tr>
<td>Heart 1 Sample 3</td>
<td>70</td>
<td>Male</td>
<td>Bypass coronary artery tied to the right coronary artery</td>
</tr>
<tr>
<td>Heart 1 Sample 4</td>
<td>70</td>
<td>Male</td>
<td>Bypass coronary artery</td>
</tr>
<tr>
<td>Heart 2 Sample 5</td>
<td>56</td>
<td>Male</td>
<td>Native anterior descendant coronary artery (highly calcified)</td>
</tr>
<tr>
<td>Heart 2 Sample 6</td>
<td>56</td>
<td>Male</td>
<td>Bypass coronary artery</td>
</tr>
<tr>
<td>Heart 2 Sample 7</td>
<td>56</td>
<td>Male</td>
<td>Bypass coronary artery</td>
</tr>
<tr>
<td>Heart 2 Sample 8</td>
<td>56</td>
<td>Male</td>
<td>Bypass coronary artery</td>
</tr>
<tr>
<td>Heart 3 Sample 9</td>
<td>56</td>
<td>Male</td>
<td>Native anterior descendant</td>
</tr>
<tr>
<td>Heart 3 Sample 10</td>
<td>56</td>
<td>Male</td>
<td>Bypass coronary artery</td>
</tr>
<tr>
<td>Heart 3 Sample 11</td>
<td>56</td>
<td>Male</td>
<td>Bypass coronary artery</td>
</tr>
</tbody>
</table>
The Ionization process was achieved with the Turbo Spray TM probe at a temperature of 400 °C at 4 kV. Acquisition of the mass spectra was performed within a scan range of 100 to 1000 Daltons with a scan rate of 1 spectrum per second. The ion count threshold was set at 1.0.

Results and Discussion

Separation of Cholesterol and Cholesteryl Esters Mixture

Optimum chromatographic resolution was obtained at room temperature using a mixture of 70% acetonitrile, 30% isopropanol, and 0.1% formic acid as the mobile phase at a flow rate of 1.0 mL/min. After the successful optimized separation of HPLC analysis of cholesterol and cholesteryl ester derivatives, calibration curves of cholesterol and cholesteryl ester were performed. The data from standard mixtures are shown in Figure 5.10.

Figure 5.10. Standard chromatogram of cholesterol and its esters using ultraviolet absorbance (λmax 200 nm). Mobile phase was a mixture of 30%/ 70% isopropanol/ acetonitrile and Bischoff Prontosil C18 column, 120 Å, 3 μm, 100 x 4.6 mm at flow rate 1 mL/m.
Calibration Standards

Figure 5.11 illustrates the calibration curve for cholesterol. A consistent linearity was obtained from 0.2 mM to 1.5 mM for cholesterol and cholesteryl esters with linear regression ($r^2$) coefficient in the range of 0.99.

![Graphs A, B, C, D showing calibration curves for cholesterol, cholesteryl linoleate, cholesteryl oleate, and cholesterol palmitate.](image)

Figure 5.11 (A-D) Calibration curves of A) cholesterol, B) cholesteryl linoleate, C) cholesteryl oleate, D) cholesteryl palmitate. The conditions are described in Figure 5.2.
**Characterization of the cholesterol and cholesteryl ester derivatives in plaque deposits by the use of HPLC-APCI-MS**

The APCI-MS results indicate that cholesterol and the cholesterol esters were present in the plaque deposits in native and bypass coronary arteries. **Figures 5.12 and 5.13** represent the UV absorbance signals and the extracted ion chromatograms at m/z=369 of crude extracts from the coronary plaque deposits from native and bypass arteries of the same heart, respectively. The ion fragment at m/z=369 corresponds to loss of water or the fatty acid side chain for free cholesterol or cholesteryl esters, respectively. **Figures 5.14A and 5.14B** illustrate the proposed ionization mechanism of cholesterol and cholesteryl esters. The presence of cholesterol and its esters in the crude extracts were confirmed using the retention times of the standard and the MS fragmentation pattern.

The quantitation of cholesterol and its esters were obtained under identical conditions. The peak areas of cholesterol and cholesteryl esters were integrated to calculate the concentrations of cholesterol and its esters present in the crude extracts, and the results are shown in **Table 5.2**. Cholesterol contributed as much as 34% and 32% in the native and bypass deposits, respectively. In contrast, the total cholesteryl esters were detected as much as 23% in the native coronary arteries and 16% in the bypass arteries.

The concentration of cholesterol was higher than the total concentrations of cholesteryl esters. The total concentrations were higher in the native coronary plaque deposits than in the bypass coronary plaque deposits. The clogged deposits in the bypass coronary arteries were made of fibrous materials. Since the deposits in the native coronary artery occur over the life span of the individual, it is likely that much more complex and crystalline materials are present. These results were consistent with the values obtained for the analysis of the carotid plaques.
using previous techniques such as nuclear magnetic resonance (NMR) and Near Infrared (NIR) spectroscopy [52, 53].

Figure 5.12 A) UV trace of crude extracted from native coronary plaque. Mobile phase was a mixture of 30%/70% isopropanol/acetoniitrile and Bischoff Prontosil C18 column, 120 Å, 3 μm, 100 x 4.6 mm at flow rate 1 mL/min. Numerous compounds beside cholesterol and cholesteryl esters were detected. B) Chromatogram of crude extracted from native coronary plaque deposits using m/z 369 as the detected fragment. Only cholesterol and cholesteryl esters were detected using peak 369.
Figure 5.13 A) UV trace of crude extracted bypass coronary plaque deposits. Mobile phase was a mixture of 30% / 70% isopropanol/acetonitrile and Bischoff Prontosil C$_{18}$ column, 120 Å, 3 µm, 100 x 4.6 mm at flow rate 1 mL/min. Numerous compounds beside cholesterol and cholesteryl esters were detected. B) Chromatogram of crude extracted from bypass coronary plaque deposits using m/z 369 as the detected fragment. Only cholesterol and cholesteryl esters were detected using peak 369.
Figure 5.14 Ionization mechanism of A) cholesterol and B) cholesteryl esters. The ionization mechanism involved proton transfer.
<table>
<thead>
<tr>
<th>Plaque deposits</th>
<th>Cholesterol</th>
<th>Cholesteryl linoleate</th>
<th>Cholesteryl oleate</th>
<th>Cholesteryl palmitate</th>
<th>Cholesteryl stearate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart 1 Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart 1</td>
<td>1</td>
<td>11 ± 0.4</td>
<td>5.1 ± 0.2</td>
<td>0.5 ± 0.1</td>
<td>13.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>32 ± 1.0</td>
<td>1.4 ± 0.1</td>
<td>5.4 ± 0.4</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.0 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>3.0 ± 0.1</td>
<td>0.02 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Heart 2 Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart 2</td>
<td>5</td>
<td>18 ± 0.6</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>13 ± 0.5</td>
<td>0.01 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>12 ± 0.5</td>
<td>5.0 ± 0.2</td>
<td>7.0 ± 0.5</td>
<td>3.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6.0 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>3.0 ± 0.2</td>
<td>*</td>
</tr>
<tr>
<td>Heart 3 Sample</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart 3</td>
<td>9</td>
<td>34 ± 2.0</td>
<td>4.0 ± 0.1</td>
<td>1.0 ± 0.1</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.0 ± 0.4</td>
<td>0.8 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>30 ± 1.0</td>
<td>4.0 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>*</td>
</tr>
</tbody>
</table>

* Not detected.
Conclusion

LC/APCI-MS analysis was successfully used to characterize native and bypass coronary plaque deposits. Fragmentation patterns and migration times were used to determine cholesterol and cholesteryl ester derivatives in the crude extracts from native and bypass coronary plaque deposits. Overall, the concentrations of cholesterol were arguably higher in the native arteries than in the bypass arteries. Surprisingly, the total concentration of cholesterol plus cholesteryl ester derivatives was less than 40% of the total plaque in all the samples analyzed. Also surprisingly, the cholesterol concentrations varied widely even within the same heart making conclusions difficult to reach. Further, the concentration of the cholesterol was always higher than the total concentration of all the esters. However, because of the small number of samples analyzed, caution must be taken in interpreting these results, which suggest further work to confirm the results here and a new study to identify the other 60% of the plaque.

References


[40] Osada, K; Ravandi, A; Kuksis, A. *JAOCS*, **1999**, 76(7), 863-871


CHAPTER 6
CONCLUSIONS AND FUTURE STUDIES

Atherosclerosis is a disease caused by a blockage of the coronary arteries as a result of an accumulation of cholesterol (and its esters), proteins, and inorganic materials onto the arterial walls. Bypass surgery, which has been one of the most efficient methods to cure the disease, remains very risky. Furthermore, the bypass veins that are used to bypass the native artery generally fill with new deposits over few years. Therefore, questions have been asked about the chemical differences between the two deposits, and the chemical forms of calcium in these deposits. To answer these questions, a combination of analytical methods such as ICP-MS, solid state NMR, and chromatography were used to characterize calcified and atheroma (amorphous) plaques from native and bypass human coronary arteries from the same heart. In chapter 2 the ICP-MS results indicated that the Ca/P ratio supported the view that the deposits existed primarily as hydroxyapatite in the crystalline plaque. However, the low total concentrations indicated that the calcium deposits were not the major component of this plaque, which appears to be organic in nature. The composition of the metal ions varied widely in the same heart. Na and Mg were higher in the soft plaque than in the crystalline plaque, and Pb concentrations were higher in the crystalline deposits. Zinc, Fe, Al, Si, Ba concentrations were very low. The concentrations of Ca and P were in mg order of magnitude higher in the crystalline deposits than in the soft plaque.

In the atheroma plaque, some hydroxyapatite appears to be present, but much of the phosphorus is organic in nature, possibly phospholipids or phosphoproteins. This may be important since the soft tissue appears to be the early form of the plaque, which
progresses to crystalline tissue over time. The organic phosphorus may be an important component in the early formation of heart plaque. By the same token, the calcium may play an equally important role at this and later stages. The low sulfur levels indicated that homocysteine may play a role in plaque formation, but is not a major component.

In Chapter 3, Part I, cross polarization magic angle spinning NMR was applied in the characterization of the plaques from native and bypass arteries. The solid state $^{13}$C CP-MAS NMR indicated that the signal from the carboxylic group was mainly due to the proteins and amino acid composition of the deposits. In addition, $^{31}$P NMR showed that in the crystalline plaque only a single phosphorus resonance was observed which overlapped with the chemical shift of the phosphorus in hydroxyapatite. Whereas, in the amorphous plaque, two main resonance peaks were observed. One correlates with the resonance chemical shift of phoshoorganic compounds and the other with hydroxyapatite.

In Chapter 3, Part II, $^{13}$C CPMAS-NMR was also applied in the studies of the interactions of Ca with homocysteine, cholesterol, and homocysteine-cholesterol. The $^{13}$C NMR spectrum of homocysteine was compared to the $^{13}$C NMR spectra of cholesterol with and without Ca. The results showed that the addition of calcium with cholesterol results in the resonance peaks of the alkene group being broader. Furthermore, the NMR of the Ca-homocysteine precipitate showed a shift of the carbon attached to the homocysteine sulfur group. Also a shift was observed at the carboxylic group in comparison with free homocysteine.

In Chapter 4, Part I, in vivo x-ray microtomography and x-ray diffraction were applied in the characterization of the crystalline and amorphous deposits from native and
bypass coronary arteries from the same heart. SR-XMT was shown to be a very competitive technique in high resolution and 3D visualization of atherosclerosis in this study. Complementary techniques such as SEM and XRD, can help to provide a complete characterization of atherosclerotic plaques. In Chapter 4, Part II, additional studies using x-ray absorption near edge structures indicated that the XANES spectra of deposits and hydroxyapatite have similar trends. However, quick EXAFS analysis of the data showed differences between the deposits and the hydroxyapatite. The results were able to provide the Ca coordination and the distances of Ca to the O.

Solid-state studies using light microscopy in combination with silver staining method on the slides were performed on the native and bypass coronary plaque deposits. The results demonstrated that the color of the stains were comparable to the one obtained with fatty acid calcium salts. In the bypass deposits Ca was evenly distributed, but in the native deposits high concentrations of Ca were found at the interface and in the arterial walls.

Finally the use of CEC and HPLC-APCI MS in Chapter 5 allowed us to determine the concentrations of cholesterol and its esters present in the deposits. First, the separation of a standard mixture of cholesterol and cholesteryl esters was optimized using isocratic capillary electrochromatography (CEC) and reversed phase high performance liquid chromatography (HPLC). Then, the same methods were applied to measure the concentrations of cholesterol and its esters in the plaque. The concentrations of cholesterol were higher in the native artery than in the bypass artery. The total concentrations of cholesterol varied widely and were less than 40% in the plaque. Furthermore cholesterol concentrations were higher than the total concentrations of
cholesteryl esters in the deposits. The results showed that more materials (phospholipids, proteins, and amino acids) were involved in the accumulation of the plaque.

**Future Studies**

Few studies have demonstrated that other chemicals in addition to cholesterol are involved in the risks of coronary diseases [1,2]. One chemical that is associated with atherosclerosis is homocysteine. It is thought that the complexation of homocysteine with Ca is a contributing factor leading to the formation of plaque deposits [3]. The results in Chapter 3, Part II, showed that Ca interacts with homocysteine and cholesterol. However, specific chemical composition was not characterized. Thus, further application using matrix assisted laser desorption mass spectrometry (MALDI-MS) would determine the structure of the complexes [4].

![Diagram](image)

**Figure 6.1 Proposed studies of the effects of homocysteine and calcium in plaque stability.**

The study of the effects of homocysteine in the oxidation of cholesterol was also proposed. Through these studies the effects of Ca on the activity of homocysteine would
be determined. The reaction products would be monitored by mass spectrometry and solid state NMR (Figure 6.1).

References


Vita


In 1985, he got a high school teaching position in The Ivory Coast (Cote D’Ivoire), where he taught mathematics and physical sciences to high school students. In 1990, he came to the United States of America. After his language training, he got a job in the Analytical Sciences Department of SmithKline Beecham, now GlaxoSmithKline. In 1994, he enrolled in the master’s program in chemistry at Pennsylvania State University. He graduated from Pennsylvania State with a master’s degree in chemistry in 1997.

After a month of thought, he entered Louisiana State University to obtain his doctorate in chemistry. Therefore, he joined the Research Group of Professor Warner because he found the work very inspiring and fascinating. Ever since, he has studied the chemical methods for the characterization of atherosclerotic plaques. His main focus was to determine the chemical differences between the native and bypass human coronary arteries from the same heart. During his academic training Serigne Thiam has published scientific papers and attended scientific meetings.

Serigne’s publications include:

Jin Hua; Ham Kyungmin; Chan Julia Y; Butler Leslie G; Kurtz Richard L; **Thiam Serigne**; Robinson, James W; Agbaria Rezik A; Warner Isiah M; Tracy Richard E. High resolution three-dimensional visualization and characterization of coronary atherosclerosis in vitro by synchrotron radiation x-ray microtomography and highly localized x-ray diffraction. PHYSICS IN MEDICINE AND BIOLOGY (2002), 47(24), 4345-56


Serigne’s presentations include:

**Thiam S**, Tittsworth CR, Rugutt JK, Warner IM *et al.*
Characterization of human atherosclerotic plaque


**Thiam S**, Shamsi SA, Rugutt JK, Warner IM *et al.*
Characterization and quantification of cholesterol and its ester derivatives extracted from heart plaque using reverse phase high performance liquid chromatography (RP-HPLC)  

**Thiam S**, Minard RD, Hatcher PG
Isolation and structure determination of lignin dimers derived from TMAH thermochemolysis of wood  

**Serigne** received the Excellence in Teaching Awards, Chemistry Department, Louisiana State University (December, 2002), the 19th International Symposium on the Separation of peptides, Proteins and Polynucleotides Travel Awards (October, 1999), the Minority Fellowship Awards Pennsylvania State University (1994), and the Analytical Chemistry Awards Analytical Sciences Department SmithKline Beecham Pharmaceuticals (1993).