High resolution three-dimensional visualization and characterization of coronary atherosclerosis in vitro by synchrotron radiation x-ray microtomography and highly localized x-ray diffraction

Hua Jin¹, Kyungmin Ham², Julia Y Chan¹, Leslie G Butler³, Richard L Kurtz¹, Serigne Thiam³, James W Robinson³, Rezik A Agbaria¹, Isiah M Warner³ and Richard E Tracy⁴

¹ Department of Physics and Astronomy, Louisiana State University Baton Rouge 70803 LA, USA
² Center for Advanced Microstructures and Devices, Louisiana State University Baton Rouge 70803 LA, USA
³ Department of Chemistry, Louisiana State University Baton Rouge 70803 LA, USA
⁴ Pathology Department, Louisiana State University Health Sciences Centre, New Orleans 70112 LA, USA

E-mail: kurtz@baton.phys.lsu.edu

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Abstract

Human atherosclerotic plaques in both native and bypass arteries have been visualized using microtomography to provide additional information on the nature of coronary artery disease. Plaques contained within arteries removed from three white males aged 51, 55 and 70 are imaged in three-dimensions with monochromatic synchrotron x-ray radiation. Fields of view are $658 \times 658 \times 517$ voxels, with cubic voxels ranging from 12 to 13 $\mu$m on a side. X-ray energies range from 11 to 15 keV (bandpass approximately 10 eV). At lower energies, high local absorption tends to generate reconstruction artefacts, while at higher energies the arterial wall is scarcely visible. At all energies, calcifications are clearly visible and differences are observed between plaques in native arteries (lifetime accumulations) versus bypass arteries (plaques developing in the interval between the heart bypass operation and the autopsy). In order to characterize coronary calcification, a micro-focused, 50 $\mu^2$, 25 keV x-ray beam was used to acquire powder diffraction data from selected calcifications. Also, large calcifications were removed from the native arteries and imaged with 25 keV x-ray energy. Calcifications are composed of hydroxyapatite crystallites and an amorphous phase. In summary, native calcifications are larger and have a higher fraction of hydroxyapatite than calcifications from the bypass arteries.

(Some figures in this article are in colour only in the electronic version)
1. Introduction

Coronary artery disease (CAD) remains the single leading cause of mortality and death in the United States and other industrialized countries (Ross 1993, Lusis 2000). Many studies have investigated methods to achieve a more accurate assessment of atherosclerosis (Sharrett 1993, Coulden et al 2000), including understanding the mechanism of this disease at a molecular and cellular level (Marotti et al 1993, Breslow 1996), as well as prevention of the build up of atherosclerotic plaques (Linton et al 1995, Duverger 1996). As the disease of atherosclerosis progresses, the structure of the plaque can change from a fibrous lesion into a complex structure containing one or more calcium-rich mineral deposits, called ‘calcifications’, embedded in a fibrous matrix (Lusis 2000). A close correlation between the extent of coronary artery calcification and CAD has been confirmed (Vogel 1988, Keelan et al 2001, Sangiorgi et al 1998, Luo et al 1997). However, detailed understanding of the three-dimensional (3D) structure of lesion has remained elusive.

The in vivo imaging methods applied to CAD have included magnetic resonance imaging (MRI) (Sharrett 1993) and MR angiography (Wasserman et al 1994), x-ray angiography (Vogel 1988), computed tomography (CT) (Pearson and Heiss 1993), electron beam angiography (EBT) (Keelan et al 2001) and ultrasonography (Maehara and Fitzgerald 2000). The calcifications are most visible with EBT, yielding two-dimensional (2D) image resolution of 0.5 mm in a 3 mm thick plane. The ultrasonographic images are able to assess both the nature of an atherosclerotic lesion such as lipid content, fibrous tissue and calcification, as well as the extent of the disease (Kritz et al 1996). The in vitro imaging methods of choice are electron and light microscopy (Fitzpatrick et al 1994, Mavrilas et al 1999, Tomazic et al 1987), as well as MRI with submillimetre spatial resolution (Worthley et al 2000, Coombs et al 2001); however, to our knowledge, no higher-resolution 3D reconstructions on atherosclerosis have been reported. The highest resolution of 3D imaging methods in atherosclerosis published is 200 µm$^3$ voxels (Coombs et al 2001). They imaged carotid atherosclerosis in vitro by using a 1.5 T clinical scanner with 3D gradient-echo MRI. By use of isotropic submillimetre resolution, the collagenous cap and underlying necrotic core typically could be distinguished and calcification could be identified. Nevertheless, this resolution is not high enough to visualize smaller plaque and, generally, calcification can not be satisfactorily visualized by MRI.

Herein, 3D images of coronary atherosclerosis plaques are presented with spatial resolutions of 9.6, 12.6 and 13.1 µm. The data sets are acquired with synchrotron radiation x-ray microtomography (SR-XMT) (Wang et al 2001, Maire et al 2001). Conventional CT versus SR-XMT has been compared by several publications (Maire et al 2001, Peyrin et al 2000). Synchrotron source offers hundreds to one thousand times higher spatial resolution than clinical medical CT scanners. The advantages of SR-XMT come from the possibility of using high photon flux, essentially monochromatic x-ray beams (Peyrin et al 2000). Monochromaticity is considered to be a key point in tomography, which is not fulfilled with conventional x-ray tubes, and prevents from beam hardening artefacts. High photon flux allows the acquisition of images at very high spatial resolution and with a good signal to noise ratio, while keeping reasonable acquisition times. In addition, the optimal energy for the examined sample may be selected in the wide energy spectrum of the synchrotron source (Peyrin et al 2000). Therefore, relative to conventional computed x-ray tomography, synchrotron x-ray sources have two significant advantages: extremely low beam divergence and intense x-ray flux density at energies covering the K-edge absorptions (Thomlinson et al 1992, Chapman et al 1995) of all elements of interest in the plaque. The low beam divergence enables efficient collection of 3D data sets without recourse to fan-beam correction as required.
in conventional CT. The wide range of available x-ray energies combined with a narrow bandpass monochromator adds a new dimension to image contrast in x-ray imaging. In vitro high-resolution, 3D imaging of atherosclerosis may provide new insights of the plaque structure.

2. Experimental details

2.1. Sample description

Human atherosclerotic arteries removed from three white males aged 51, 55 and 70, denoted A, B and C, respectively, were measured. The arteries were obtained from the Pathology Department of the Louisiana State University Medical Center in New Orleans. They were kept frozen prior to the SR-XMT measurements, though experiments were done with samples warmed to room temperature. Two kinds of arteries, native and bypass, were used in this study. The native coronary artery deposits are associated with the materials that are accumulated during the life span of the patient. In contrast, the bypass plaque deposits are exclusively accumulated after the bypass surgery. Therefore, chemical differences between the two plaques may be exhibited due to changes in body metabolism, or factors such as diet, lifestyle and age. The elapsed time between the bypass operation and death is not available to the authors.

Figure 1 illustrates the schematic drawing of a native artery and bypass. The native arteries of the A and B specimens were from the left circumflex and the C sample was from the anterior descending coronary artery. Each artery was cut to about 20 mm in length and inserted longitudinally into a plastic straw and sealed to prevent dehydration.

2.2. Synchrotron radiation x-ray microtomography instrumentation and data handling

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. Monochromatic x-ray energies of 11–15 keV were evaluated for the arteries to maximize contrast. Large pieces of calcifications were separated from the native arteries and imaged with 25 keV monochromatic x-ray energy.

For the SR-XMT measurements, the transmitted x-rays were converted to visible light using a synthetic garnet (Ce:YAG) scintillator. The visible light from the scintillator was imaged with a 5 × microscope objective with 25 or 50 mm tube extender onto a high-speed 12-bit CCD camera (Princeton Instruments Pentamax with 1317 × 1035 pixels; Roper Scientific MicroMAX 5 MHz with 1300 × 1100 pixels). With the optical magnification and 2 × 2 software binning, the effective pixel size was 13.1 µm for arteries A and B, 12.6 µm for artery C and 9.6 µm for the calcification-only samples. 720 images were taken between 0 and 179.75 degrees in 0.25 degree increments for each sample.
Figure 2. Translucent 3D visualization of the B (55 yr male) coronary artery. (a) Native artery. The vertical direction is the longitudinal direction and the field of view is 6.4 × 6.4 × 3.9 mm³. This rendering shows the sample holder, artery wall and calcifications. (b) Bypass artery. The field of view is 6.4 × 6.4 × 6.8 mm³. This rendering shows a calcification in the bypass artery within the sample holder.

From a series of acquired projections, a tomogram was reconstructed in the IDL (interactive data language: http://www.rsinc.com/idl/) programming environment using a fast Fourier transform algorithm following re-gridding from polar to cartesian coordinates (http://cars9.uchicago.edu/software/tomography). Typical reconstructed volumes for the arteries are 658 × 658 × 517 voxels in size, corresponding to fields of view of 8.3 × 8.3 × 6.5 mm³ or slightly larger. When stored as scaled 16-bit integers in netCDF format, the data file sizes are about 0.43 GB. The reconstructed volumes contain the x-ray mass attenuation coefficient at each voxel. Further 3D visualization and image analysis were done using IDL.

2.3. X-ray diffraction of calcification

In situ x-ray diffraction (XRD) patterns were acquired with the same beamline by adjusting slits to create a 50 µm square beam. The incident monochromatic x-ray energy was 25 keV (λ = 0.496 Å). With a 2 min exposure, the powder diffraction pattern was recorded on an image plate and analysed with FIT2D software to yield a 2-theta scan (http://www.esrf.fr/computing/expg/subgroups/data_analysis/FIT2D/).

3. Results and discussion

3.1. 3D visualization of coronary atherosclerosis

Figure 2 shows translucent images of the B (55 yr male) native and bypass arteries as examples of 3D visualization. The structures from outer surface through the interior native artery that includes organic tissue and calcification, which correspond to low, medium and high-mass
High resolution 3D visualization and characterization of coronary atherosclerosis

3.2. Crystalline structural characterization of the coronary calcifications

The high-density plaque was demonstrated to contain calcium deposits by in situ powder XRD measurements. Figure 3 depicts typical x-ray powder diffraction patterns of the native and bypass samples obtained at a wavelength of 0.496 Å. Deposits in native arteries were far more crystalline than in bypass arteries. By integrating the Debye–Scherrer rings the equivalent 2θ scan diffraction patterns were obtained, as shown in figure 3(c). Each peak can be indexed according to the hexagonal space group P6$_3$/m, consistent with the cell ($a = 9.424$ Å, $c = 6.879$ Å) previously published (Sudarsanan and Young 1969). No unidentified peaks are observed, suggesting that the crystalline component of calcified plaque is calcium hydroxyapatite (Ca-HAP) with the chemical formula Ca$_{10}$(PO$_4$)$_6$(OH)$_$_2$. The broadening
3.3. 3D visualization of coronary calcification

Figure 4(a) is a 3D visualization of just the calcifications in the native and bypass arteries of the three patients. Apparently, calcifications in native arteries are much larger than those in bypasses and only a few small calcifications were found in the bypass arteries. Many of the calcifications in the native arteries were preferentially deposited in closed cylinder-like shapes along the longitudinal axis of the artery, especially for large accumulations. Not all large deposits were in the closed cylinder-like forms. Some had arc-like shapes confined by
Figure 5. Experimentally measured linear voxel attenuation coefficients $\mu$ of the calcified plaques. The line and number in each image indicate scan route of the profile and the slice number, respectively. The vertical axes on the right-hand side of each figure illustrate the $\mu$ tick mark values (left axis) equivalent to percentages of the attenuation coefficient of pure Ca-HAP. The NIST values of Ca-HAP mass attenuation coefficients $\mu$ at various x-ray energies indicated in the figures were obtained by multiplying $\mu/\rho$ (table 1) and $\rho$ of Ca-HAP (3.14 g cm$^{-3}$). (a) Calcification-only removed from each native artery. The monochromatic x-ray energy was 25 keV. (b) Calcification within each bypass. The x-ray energies were 12.5, 12.5 and 15 keV for A, B and C, respectively. Origins of the right vertical axes were drawn at the attenuation from the fibrous plaque which contains no Ca-HAP crystals.

3.4. Percentage Ca-HAP

The mass attenuation coefficient $\mu/\rho$ is defined by the well established Bouguer–Lambert–Beer exponential absorption law (Bouguer 1729, Lambert 1760, Beer 1852)

$$I/I_0 = \exp\left[-\left(\frac{\mu}{\rho}\right)x\right],$$

(1)

in which $I_0$ is incident intensity of a narrow beam of monoenergetic photons, $I$ is attenuated intensity from $I_0$ in passing through a layer of material with mass per unit area $x$. The attenuation coefficient $\mu/\rho$ can be obtained from measured values of $I_0$, $I$ and $x$ data by rewriting equation (1) as

$$\frac{\mu}{\rho} = -x^{-1}\ln\left(\frac{I}{I_0}\right).$$

(2)

Figure 5(a) depicts three experimental linear attenuation coefficient profiles of the calcification-only removed from the native arteries, measured 25 keV. The average attenuation coefficients of these three calcifications range from 4.4 to 4.8 cm$^{-1}$. The plateaus at 1.1 cm$^{-1}$ are attenuations from the sample holders. Because the calcifications in the bypass arteries were too small to
Table 1. Attenuation coefficient $\mu/\rho$ for each component at various x-ray energies. The values were from the website of National Institute of Standards and Technology.

<table>
<thead>
<tr>
<th>$E$ (keV)</th>
<th>$(\mu/\rho)_{\text{HAP}}^*$ (cm$^2$ g$^{-1}$)</th>
<th>$(\mu/\rho)_{\text{Tissue}}^{**}$ (cm$^2$ g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>36.1</td>
<td>3.8</td>
</tr>
<tr>
<td>12.5</td>
<td>25.1</td>
<td>2.6</td>
</tr>
<tr>
<td>13.5</td>
<td>20.2</td>
<td>2.1</td>
</tr>
<tr>
<td>15</td>
<td>14.9</td>
<td>1.6</td>
</tr>
<tr>
<td>25</td>
<td>3.5</td>
<td>0.6</td>
</tr>
</tbody>
</table>


Table 2. Fitted contributions of Ca-HAP and soft tissue to the measured $\mu$ values in the calcified plaque.

<table>
<thead>
<tr>
<th>Contribution</th>
<th>At $\mu_{\text{ave}}$ in native artery (%)</th>
<th>At $\mu_{\text{max}}$ in bypass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca-HAP</td>
<td>A 38</td>
<td>A 19</td>
</tr>
<tr>
<td></td>
<td>B 38</td>
<td>B 28</td>
</tr>
<tr>
<td></td>
<td>C 38</td>
<td>C 30</td>
</tr>
<tr>
<td>Tissue</td>
<td>A 62</td>
<td>A 81</td>
</tr>
<tr>
<td></td>
<td>B 62</td>
<td>B 72</td>
</tr>
<tr>
<td></td>
<td>C 62</td>
<td>C 70</td>
</tr>
</tbody>
</table>

extract, their experimental attenuations were examined along with the bypass arteries at 12.5 keV or 15 keV monochromatic x-ray energies as shown in figure 5(b). The maximum calcification attenuations are 15.5, 22.3 and 12.7 cm$^{-1}$ for the bypasses A, B and C, respectively. In order to compare our experimental results $\mu$ with NIST data (http://physics.nist.gov), the x-ray attenuation coefficient $\mu/\rho$ for pure Ca-HAP and soft tissue are listed in table 1. The densities are 3.14 g cm$^{-3}$ and 1.0 g cm$^{-3}$ for Ca-HAP and soft tissue, respectively. In figure 5(b), the plateaus at 2.4 cm$^{-1}$ for A and B, and at 1.4 cm$^{-1}$ for C are attenuations from the tissues, which are close to that of the soft tissues at 12.5 keV and 15 keV, respectively (table 1). The right-hand side vertical axes of figures 5(a) and (b) illustrate the equivalent experimental $\mu$ values of the left vertical axes to the percentages of pure Ca-HAP crystal at various x-ray energies.

Assuming the calcified plaque was composed of Ca-HAP only crystals and connective tissue (neglecting the trace amount of metals Mg, Na and Fe (Tomazic et al 1987, Murungi and Robinson 1992)), we estimated the attenuation contribution fraction $\chi$ of Ca-HAP crystals in the calcifications by equation $\mu_{\text{exp}} = \chi \times \mu_{\text{HAP}} + (1 - \chi) \times \mu_{\text{Tissue}}$, the results are given in table 2. Based on the average attenuation coefficient, the calcifications in the native arteries contain approximately 38% Ca-HAP, with the balance 62% tissue. The bypass arteries have less dense calcifications, consisting of 19–30% Ca-HAP.

3.5. Plaque components

Figures 6(a) and (b) show representative cross-sectional images of the coronary arteries. Within each row, the native and bypass arteries were from the same heart. Slice number is indicated in the upper right corner of each image.

Three types of plaque components were distinguished by brightness in these samples, indicating the low, medium and high mass densities, consistent with the previously employed intravascular ultrasound imaging method (Vallabhajosula and Fuster 1997). The low-density
Figure 6. Cross-sectional image sequences along the three different directions. (a) Transaxial slices. Four slices of each native artery are shown in the left column; the right column shows one CT slice of each bypass. The sample holder (uniform plastic cylinder), artery wall, low, medium and high mass density components are indicated as white arrowhead, white diamond, white arrow, white star and black arrow, respectively. (b) Two slices of each native artery along the x-axis (the left two columns) and y-axis (the third and fourth columns). The right two columns depict one slice of each bypass along the x- and y-axis, respectively. (c) Absorption along the bypass (dashed line) and native (solid line) arteries of B. These lines span all three plaque phases and air. Three lines were drawn as eye guides for the attenuations from calcified, fibrous and fatty plaques, respectively. The right vertical axis illustrates the $\mu$ tick mark values (left axis) equivalent to percentages of the attenuation coefficient of pure Ca-HAP. Its origin was drawn at the attenuation from the fibrous plaque.
component has been identified as lipid-rich deposits; medium as fibrous plaque and high mass density particles as calcified plaque. These three phases were observed by the profile of attenuation coefficient $\mu$ as shown in figure 6(c). The attenuation by fibrous plaque of 12.5 keV x-rays is about 2.5 cm$^{-1}$, twice that of fatty plaque. The attenuation coefficient of the fibrous plaque is very close to that of soft tissue, 2.6 cm$^{-1}$. The calcium deposit has around ten times higher absorption than the fibrous plaque. The cross-sectional images of the native arteries clearly show the 3D morphologies and distributions of the plaque components in atherosclerotic arteries. Some calcified plaques were separately distributed as small clusters while some are large continuous pieces along the vessel axis. The density of the calcification edges was larger than that of the interiors, which is also seen from figure 6(c). However, this phenomenon did not exist in the calcification-only experiments which were run at 25 keV energy (see figure 5(a)). The intensity differences between the edges and interiors of the calcifications in the native arteries done at 11–15 keV monochromatic energies may be reconstruction artefacts. At lower energies, high local absorptions tend to generate reconstruction artefacts.

It can be seen from figure 6(a) that the bypass arteries are blocked by fibrous plaque instead of calcifications. These blockages are due to bulky fibrosis related to wound healing and scar formation. They have some resemblance to restenosis of arteries in the weeks following angioplasty procedures of various types. This dense fibrosis becomes a ready substrate for fatty degeneration and eventual calcification.

4. Conclusion

In this study, SR-XMT has proven to be a very effective technique providing high resolution and 3D visualization of atherosclerosis. In conjunction with SEM or XRD, one can obtain the structure of atherosclerotic plaques. Although the spatial resolution of SEM is higher than that of SR-XMT, the field of view of SEM is smaller and inherently 2D. For this reason, SR-XMT fills a very important void, bridging the microscopic to mesoscopic length scale, and allows us to study the interior of materials in three dimensions.

The calcified plaque was identified as Ca-HAP crystals with small diameters distributed in a tissue matrix. By fitting the attenuation coefficients of Ca-HAP and soft tissue, we estimated that the average relative concentration of Ca-HAP crystals in native calcification was 38%, while the highest relative concentration in bypass calcification was 19–30%, with the balance likely consisting of soft tissue. Native arteries contained much more calcified plaque than the bypasses, several orders of magnitude more. For most of the large calcium accumulations, the calcifications in native arteries were deposited in closed cylinder-like shapes along the arteries, while some calcifications had arc-like shapes confined by artery wall. Three types of atherosclerotic plaque components were observed, recognized as fatty, fibrous and calcified plaque, respectively.

Acknowledgments

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Endnotes

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