Reflection spectroscopy of atherosclerotic plaque

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Abstract. Heart disease is the primary cause of death in the western world. Many of these deaths are caused by the rupture of vulnerable plaque. Vulnerable plaques are characterized by a large lipid core covered by a thin fibrous cap. One method for detecting these plaques is reflection spectroscopy. Several studies have investigated this method using statistical methods. A more analytic and quantitative study might yield more insight into the sensitivity of this detection modality. This is the approach taken in this work. Reflectance spectra in the spectral region from 400 to 1700 nm are collected from 77 measurement points from 23 human aortas. A measure of lipid content in a plaque based on reflection spectra is presented. The measure of lipid content is compared with the thickness of the lipid core, determined from histology. Defining vulnerable plaque as having a lipid core >500 μm and fibrous cap <500 μm, vulnerable plaques are detected with a sensitivity of 88% and a specificity of 94%. Although the method can detect lipid content, it is not very sensitive to the thickness of the fibrous cap. Another detection modality is necessary to detect this feature. © *2006 Society of Photo-Optical Instrumentation Engineers.*
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1 Introduction

Atherosclerotic plaques can be dangerous in two ways. First, the degree of stenosis can be severe enough to obstruct blood flow, thereby causing hypoxia in the tissue distal to the obstruction. Second, a plaque can rupture, exposing the blood stream to thrombogenic material that can induce the formation of a thrombus and sudden occlusion of the vessel. The latter is more dangerous because it is usually nonsymptomatic before rupture, and currently there exists no detection modality in clinical use that can detect these plaques. Plaques that are prone to rupture are denoted as vulnerable plaques. They are characterized by a large lipid core covered by a thin fibrous cap. Felton et al. reported the average lipid composition of the plaque as 25% free cholesterol, 52% cholesterol esters, 14% phospholipids, and 9% triglycerides.[1] Blankenhorn et al. have shown that the concentration of carotenoids (mainly beta-carotene) is larger in lipid-rich plaque than in a normal aorta.[2] Vulnerable plaques also have a thin fibrous cap, leaving them mechanically unstable. Virmani et al. give a value for the lipid core as less than 65 μm for a plaque to be denoted vulnerable.[3] Prediman reports a cap thickness of less than 40% of total plaque size as a marker for vulnerability.[4] This work investigates the use of reflection spectroscopy for detecting the lipid core, and whether it is possible to quantify the thickness of the lipid core and fibrous cap.

Many other methods have been proposed for the detection of vulnerable plaques, e.g., fluorescence spectroscopy, Raman spectroscopy, elastography, thermography, and intravascular ultrasound (IVUS). Methods that are in clinical use today for detecting plaque (angiography, IVUS) cannot differentiate between vulnerable and stable plaques.

Existing studies on the applicability of reflection spectroscopy for detecting plaque can roughly be separated in two groups. It has been shown that near-infrared reflection spectroscopy (NIRS) correlates with measurements of the chemical composition of the plaque.[5] It has also been shown that NIRS can differentiate between vulnerable and stable plaques, as determined from histology with good sensitivity and specificity.[6–8] This has been shown using statistical methods like principle component analysis (PCA)[6,7] and cluster analysis.[8]

In this work, a more analytic approach is taken. The region where a change in the reflection spectrum is expected is determined from the absorption spectra of the known constituents of plaque. It is determined if the change in spectral features can quantitatively be correlated with lipid content in the plaques. The major chromophores in the near-infrared (NIR) region are water, lipid, and cholesterol. The absorption spectra of these substances are shown in Fig. 1.[9] Fat has three absorption peaks in the NIR region at approximately 930, 1200, and 1390 nm. The first two bands corresponds to the third and second overtone of the C-H stretching vibration. The third band is a combination of stretching and deformation vibrations of the C-H bond.[10] Wang et al. reported absorption peaks at 1210, 1720, and 2304 nm.[8]

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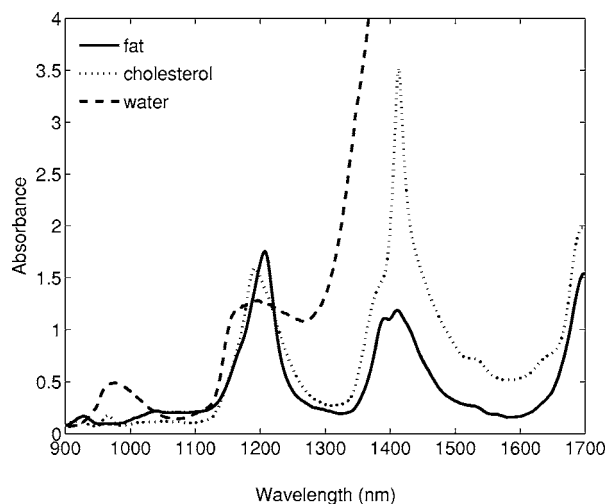
Fig. 1 Absorption spectra of the most important absorbers in the NIR spectral region.[9]

Fig. 3 A schematic view of the tip of the two fiber probes used in the experiment. The white circles represent excitation fibers and the gray represent collection fibers.

In the visible region, the most significant change in the spectrum for lipid-rich plaques is probably due to a higher concentration of carotenoids (mainly beta-carotene).[2] Figure 2 shows the absorption spectrum of beta-carotene.[11] Based on the knowledge of this spectrum, regions where there is a change in the reflectance spectrum due to lipid content in the plaque has been identified. A simple measure of lipid content in the plaque based on this information is proposed.

2 Materials and Methods

2.1 Materials

A section of the thoracic aorta from 23 patients (14 men and 9 women) were excised during autopsy. No special rules were employed in selecting the patients that were included in the study. Age of patients at death were 56 ± 21 years. In the time between autopsy and measurements ($\sim$2 h), the sample was kept in phosphate buffered saline (PBS) at room temperature. The section of the aorta was opened longitudinally and placed
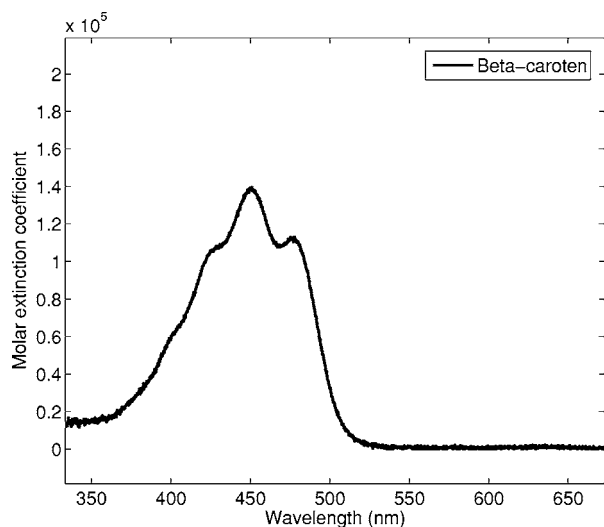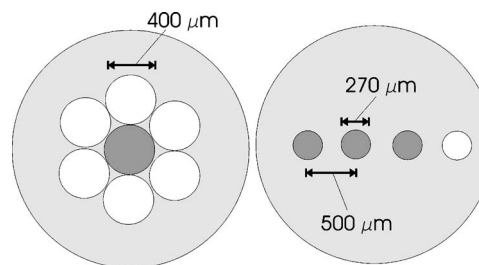

Fig. 2 Molar extinction coefficient of beta-carotene.[11]

on a piece of black cardboard with the lumen side facing up. For two of the samples, measurements were also collected with white copy paper as the bottom layer, but no systematic difference in the reflectance was detected. Between one and seven measurement points were selected from each sample, depending on the prevalence of atherosclerotic plaque. The selection was performed by visual inspection. One measurement point was chosen from a region that looked normal and the remaining points from areas that looked like plaque. The entire measurement series took about 2 h for each sample. During measurements, the samples were kept hydrated by dripping PBS on the samples with a pipette. Just before placing a probe on the sample, the surface was lightly dried with paper tissue.

2.2 Methods: Reflection Measurements

Reflection spectra in the wavelength range 400 to 1700 nm were acquired for each measurement point using two different spectrometers (Ocean Optics SD2000, double channel; and Ocean Optics NIR512, Ocean Optics, Duiven, The Netherlands). The SD2000 spectrometer is sensitive in the region from 400 to 1100 nm, and the NIR512 spectrometer from 900 to 1700 nm. Three different probes were used in the measurements: 1. an integrating sphere (Ocean Optics, ISP-REF), 2. a fiber reflection probe (Ocean Optics, VIS-NIR) with seven excitation fibers placed in a circular fashion around a central collection fiber (hereby denoted "circular probe"), and 3. a fiber reflection probe (Ocean Optics, custom design) with four fibers placed in a linear array (hereby denoted "linear probe"). For the linear probe, the fiber furthest to the side was used as the excitation fiber, and the three other fibers could act independently as collection fibers. For a schematic drawing and dimensions of the fiber probes, see Fig. 3. All the probes were connected to the spectrometers via fiber connections.

The integrating sphere was hand-held during measurements while the fiber probes were positioned using a micrometer positioning stand and kept just in contact with the tissue during measurements. The contact point was determined by visual inspection. The sample was not fixed to the stand, so the placement of different probes at the same point was done visually. The error of the lateral position was estimated to be less than 2 mm.

Spectra were recorded as percent reflection relative to a reflection standard (SRS-99-010, LabSphere, North Sutton, United Kingdom). The integrating sphere was placed in direct contact with the standard for the reference measurements. For
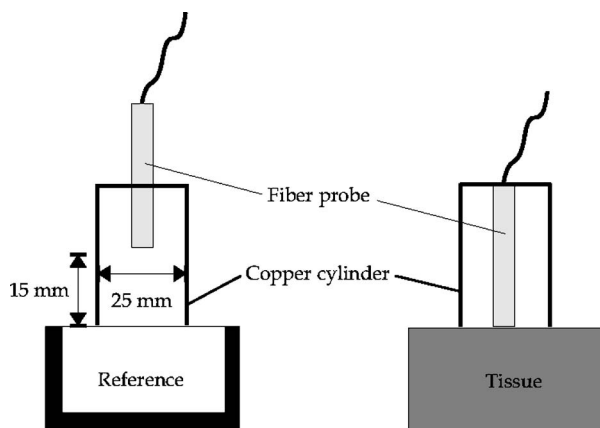
Fig. 4 A schematic view of the reference measurement used for the fiber probes. For the reference measurement (left), the fiber probes were positioned 1.5 cm away from the standard but were in contact with the tissue during reflection measurements (right).
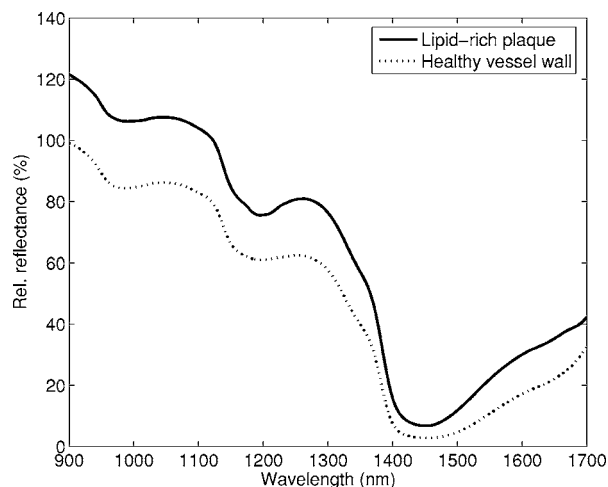

Fig. 5 Example reflectance spectra from the NIR region. For the lipid-rich plaque (solid line), there is an additional local minimum around 1200 nm that is superimposed on the broader minimum due to water absorption.

the circular and linear probes, the tip of the probe was positioned 15 mm from the surface of the reflectance standard. The reflectance standard has a very high scattering coefficient, so the penetration depth is very small. If the fiber probes are put on the surface, very few photons will reach a fiber that is separated from the excitation fiber by more than one penetration depth. This effect might be wavelength dependent, and this is the reason that the fibers were positioned away from the surface of the reflectance standard. The reflectance values for the probes are thus not relative to 100% reflectance, but relative to a spectrally flat standard of unknown absolute reflectance. A black-painted copper cylinder was placed around the probe to shield from ambient lighting. The same black cylinders were used during measurements to shield from the ambient lighting, but the probes were then in contact with the tissue. The procedure is illustrated in Fig. 4.

2.3 Methods: Histology

The samples were prepared for histology immediately after the spectroscopic measurements. A rectangle ($\sim 10 \times 4$ mm) was cut out around the measurement point with a scalpel and divided along the long center line. One part was fixed in 10% buffered formalin and embedded in paraffin, while the other was frozen in liquid nitrogen. The samples were then sliced and stained. The formalin sample was stained with hematoxylin-eosin-saffron (HES) and the frozen sections with Sudan(3). The sections were then photographed through a microscope at three different magnifications (2, 10, and 20$\times$). To be able to quantify the size of histological sections, a microscopic measurement stick was also photographed at the various magnifications. The size of the histological features (total plaque, lipid core, and cap thickness) was measured by manually comparing prints of the sample and the measurement stick, at the same magnification, using a ruler.

3 Analysis

3.1 Spectral Analysis

The first derivative of the NIR spectra was computed using the method of Savitzky and Golay using the software package

Matlab (Mathworks, Natick, MA).[12] The lipid peak around 1200 nm causes a local minimum in the spectrum that is superpositioned on the reflectance minimum due to the water absorption (see Fig. 5). The derivatives of these spectra are shown in Fig. 6. For the normal vessel, the derivative increases monotonically (i.e., a decrease in the negative slope of the reflectance spectra), while for the lipid-rich plaque, the derivative starts to decrease around 1168 nm due to the superpositioning of the lipid absorption band over the water absorption band. The difference between the peak and the valley of this spectrum is assigned as the lipid index denoted C_l. This is illustrated in Fig. 6.
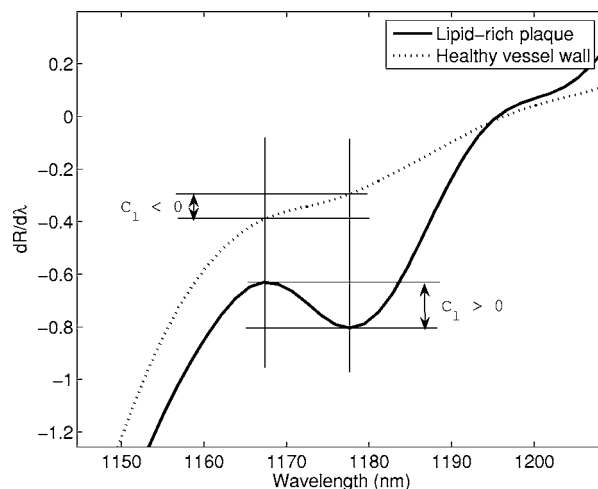

Fig. 6 Illustration of the lipid index $C_l = dR/d\lambda|_{\lambda=1168} - dR/d\lambda|_{\lambda=1178}$. The figure shows the derivative of the spectra in Fig. 5. For the normal artery (dashed line), the derivative increases monotonically, whereas for the lipid-rich, there is a local minima due to the superimposed lipid absorption. $C_l > 0$ when a certain amount of lipid is present in the sampling region of the probe.
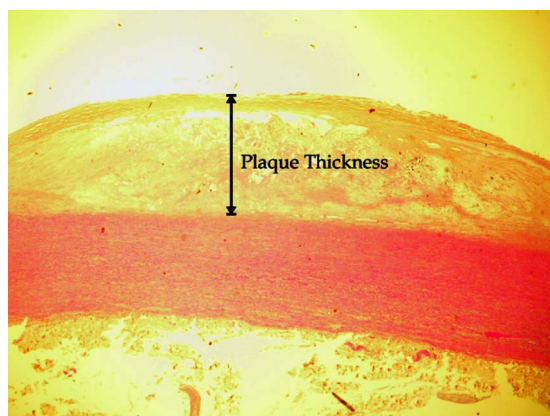
Fig. 7 Definition of plaque thickness.

Fig. 8 Definition of lipid core thickness and fibrous cap thickness.

$$C_l = \frac{dR}{d\lambda}\bigg|_{\lambda=1168} - \frac{dR}{d\lambda}\bigg|_{\lambda=1178}. \tag{1}$$

In the visible region, the lipid-index C_l' was taken as the difference between the reflection at 560 nm (where there is little beta-carotene absorption but substantial blood absorption) and the reflection at 450 nm (the absorption peak of beta-carotene), divided by the reflection at 700 nm. At 700 nm, the absorption of both beta-carotene and blood is relatively small. Dividing by this value accounts for the overall reflectance level of the spectrum due to scattering properties.

$$C_l' = \frac{R(560) - R(450)}{R(700)}. \tag{2}$$

3.2 Histological Analysis

A picture was taken of a microscopic measuring stick for each magnification so that absolute lengths could be determined in the histological photographs. From these photographs, the thickness of the total plaque, the fibrous cap, and the lipid core were determined. The total plaque thickness was determined from the HES stained sections as the distance from the media (defined as the presence of red colored elastin fibers) to the endothelium (Fig. 7). The core thickness was determined from the Sudan Red stained sections as the thickness of the area where red stained lipid droplets were present. The fibrous cap thickness was defined as the distance from the inner lining of the vessel to the start of the lipid core (Fig. 8).

4 Results

Figures 9 and 10 contain typical reflectance data from an artery for the NIR and visible spectral region. The corresponding aorta sample is shown in Fig. 11. The only change in the NIR reflectance spectra where there is known lipid absorption was at a point around 1200 nm. In the visible region, there seemed to be an increased absorption from 450 to 500 nm for lipid-rich plaques.

The spectral analysis, defined in Sec. 3.1 was then compared with the histological studies. In Figs. 12–15, plots of C_l and C_l' are plotted against the lipid core thickness. In these plots, plaques with a lipid core greater than 500 μm or a
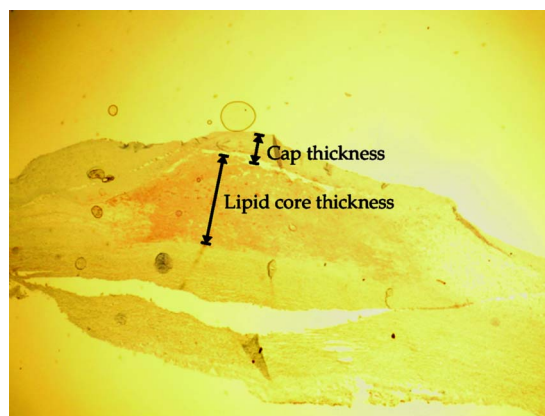
fibrous cap less than 500 μm are defined as vulnerable. For measurements in the NIR, a line has been drawn at $C_l=0$ as a possible demarcation between a lipid-index, indicating a stable or vulnerable plaque. For the visible region (Fig. 15), the demarcation line was arbitrarily put at 0.25, which seemed to yield the best results. Only data for the circular probe are presented for the visual region. Using these definitions, the sensitivities and specificities for the various measurements were calculated. The results are tabulated in Table 1.

5 Discussion

Water is an important absorber in the NIR region. Even though the samples were put in PBS, which has the same osmotic pressure as tissue, there might be some absorption of water into the tissue, which can cause swelling. This might change the optical properties of the tissue. However, the endothelium of the aorta is constantly exposed to the liquid environment of the blood stream, so it seems likely that the sample should be somewhat resistant to this effect. On the other hand, since the combined measurements took up to 2 h, there might be some dehydration during the experiment, even if PBS was often dripped onto the sample. This swelling or dehydration effect (whichever is stronger) might affect the reflectance spectra on which the calculation of C_l is based.

The thickness of the vessel wall was different as determined from the formalin fixed sections compared to the frozen sections. Size artifacts are known problems in histology.[13] The measurement of the lipid core thickness might therefore have an inherent systematic error. The precision of the size measurements was estimated to be about 50 μm. This was calculated as the average size difference of the same structure, measured in the frozen and in the formalin fixed sections. This gives no estimate on how well the measurements compare to the original size of the plaque, as there might be other systematic errors linked to the fixation process.

From Fig. 10, it seems like the difference in the spectra between a normal vessel and a lipid containing plaque is stronger in the visible region than in the near-infrared region. However, Fig. 15 shows that the correlation with lipid content is poorer. This is most likely due to the large blood absorption that overlaps the absorption spectra of beta-carotene. Small differences in the vasculature of the vessel can have large implications on the measured spectra. This can be seen in Fig.
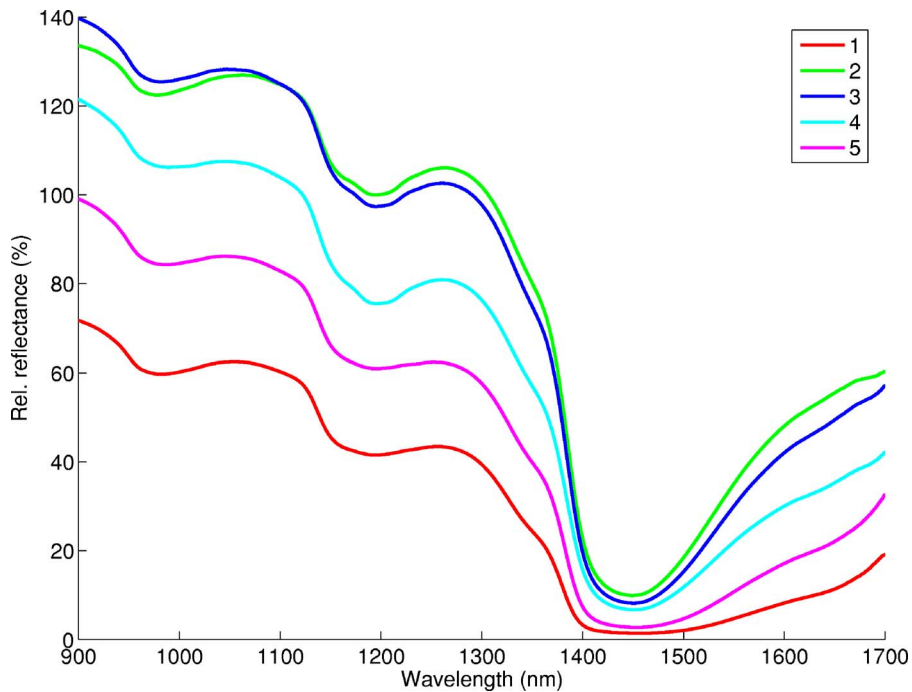
Fig. 9 Reflectance spectra in the NIR region corresponding to the measurement points in Fig. 11.

16, where two reflection spectra of normal aorta from a 24 and a 78 year old male are shown. In general, the older the person was from which the aorta was taken, the redder the vessel appeared. This variation had a stronger effect on the spectra the deeper the penetration depth of the probe was. No trends could therefore be seen for the integrating sphere and the linear probe, and they have hence not been included. Also, the large blood absorption for wavelengths below 700 nm makes it difficult to perform measurements through even a thin film of blood, making it uncertain if a measurement is feasible. Some form of flushing of the artery is most likely needed. Since carotenoids are preferentially absorbed in lipid-rich plaque, it might be possible to attach some fluorescent molecule or stronger chromophore to a carotenoid or other lipophilic molecule to increase the contrast between stable and vulnerable plaque. The preferential uptake of endogenous beta-carotene in plaque has been demonstrated by Prince, Glenn, and MacNichol.[14]

The absorbance values in Fig. 1 are measured in pure form. The absorbance peaks will change slightly due to the chemical environment, so these spectra cannot be taken exactly as the absorbance values that are expected from the tissue. Neither are the many types of lipids in the plaque the same as those used in the absorbtion measurements.[1] The absorbance spectra should only be regarded as indications of where variations in the reflectance spectra can be expected.

A histological section of a vulnerable plaque can be seen in Fig. 7. It is apparent that the thickness of the fibrous cap
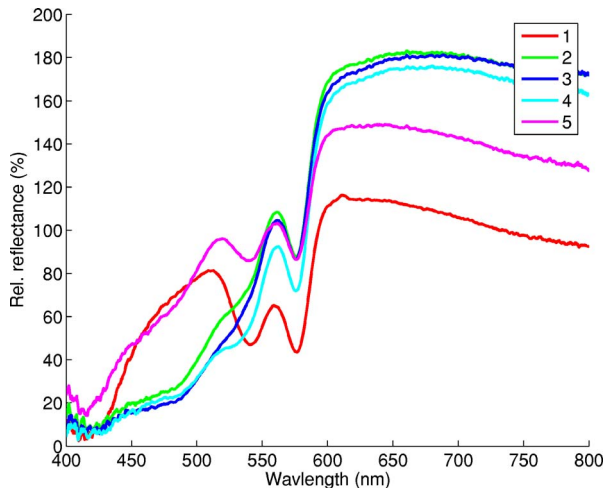

Fig. 10 Reflectance spectra in the visible spectral region corresponding to the measurement points in Fig. 11.
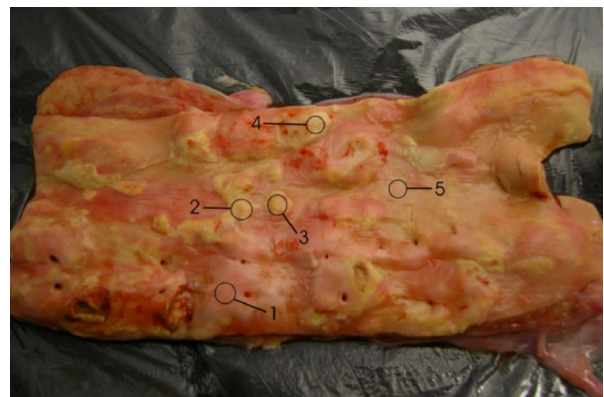

Fig. 11 Aorta sample. The circles show measurement points and the numbers correspond to the reflectance spectra in Figs. 10 and 9. The sample is approximately 15 cm long.
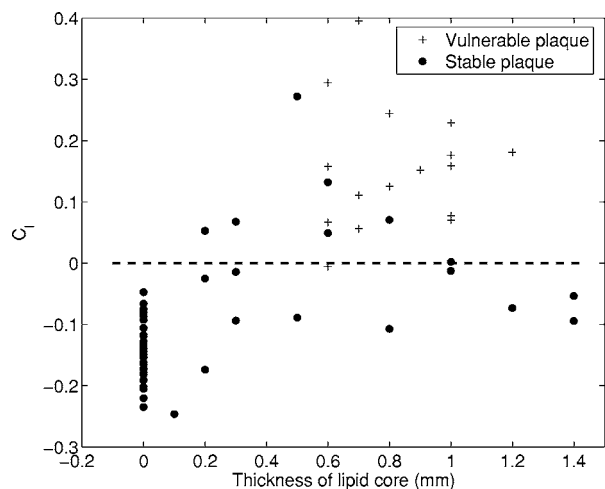
Fig. 12 Lipid index C_l versus thickness of lipid core, measured with the circular fiber probe.

Fig. 14 Lipid index C_l versus thickness of lipid core, measured with the integrating sphere.

varies over the extent of the plaque. Any clinical implementation of this method will need to have a lateral resolution less than 1 mm, or vulnerable plaques might be incorrectly characterized as stable even though they have a very thin cap on the edge, where the mechanical forces are strongest.

The spectroscopic measurements are to a certain degree sensitive to the thickness of the cap, as can be seen in Figs. 12 through 15. Even though a plaque has a large lipid core, it gives a low value of C_l, since it is covered by a thick fibrous cap. However, in the literature a plaque with cap thickness less than 65 μm is defined as a vulnerable plaque, and this is not detectable with the current instrumentation.[3] The definition used in this work is partially based on the discrimination that could be expected, given the geometry of the probes that were used, and partially warranted by the definition given in one of the references.[4] If the minimum cap thickness of 65 μm had been used, many points in the upper right quadrant of Figs. 12 through 15 would be considered stable instead

of vulnerable. If plaques with lipid cores less than 500 μm had been considered vulnerable if the cap was thin enough, many points in the lower left quadrant of Figs. 12 through 15 would be vulnerable instead of stable. The definition used was chosen to show that the method detects the presence of lipid and is also partially sensitive to the thickness, but that probably an additional method with high resolution is needed to measure the cap, e.g., optical coherence tomography, confocal microscopy, or multiphoton microscopy. An array of very small fibers ($\sim$10 μm) with very small fiber separation could also achieve this. There are also lipid absorption peaks higher up in the infrared spectrum where the penetration depth is small due to the high water absorption. Using several wavelengths with different penetration depths, it could be possible to calculate approximately at what depth the lipid core is. A tomographic method that can also detect the lumen surface is probably best, since the precise positioning of a probe inside a coronary artery is difficult. A sensor using reflection spectroscopy could be used to scan the artery. If the presence of lipid
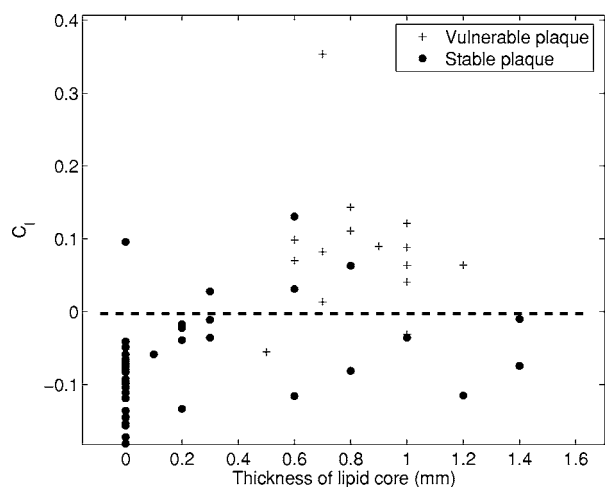

Fig. 13 Lipid index C_l versus thickness of lipid core, measured with the linear fiber probe, using the fiber nearest to the excitation fiber as the collection fiber. Using the other fibers as collection fibers gave poorer correlation with the thickness of the lipid core.
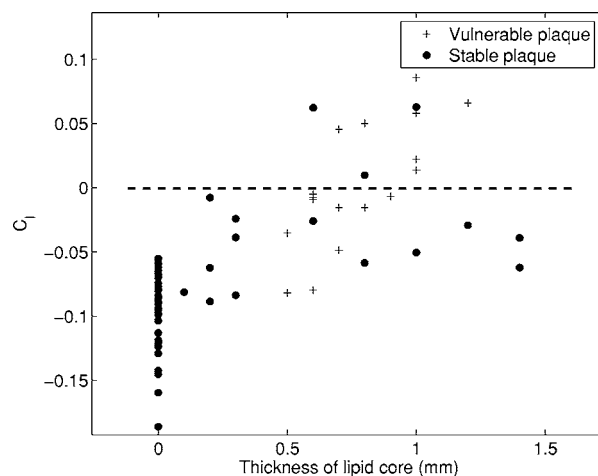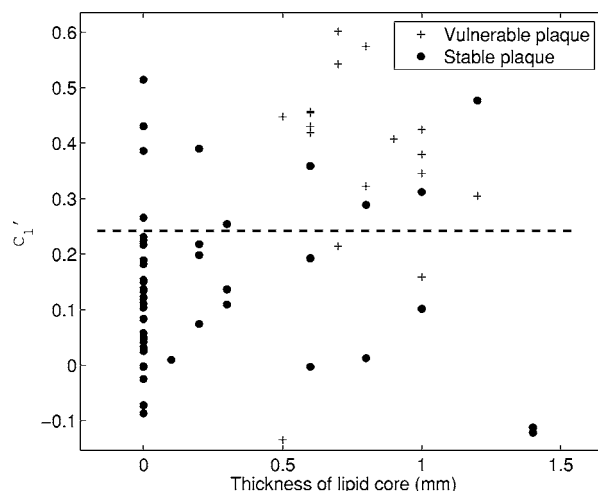

Fig. 15 Lipid index C_l' for the visible region versus thickness of the lipid core, measured with the circular fiber probe.
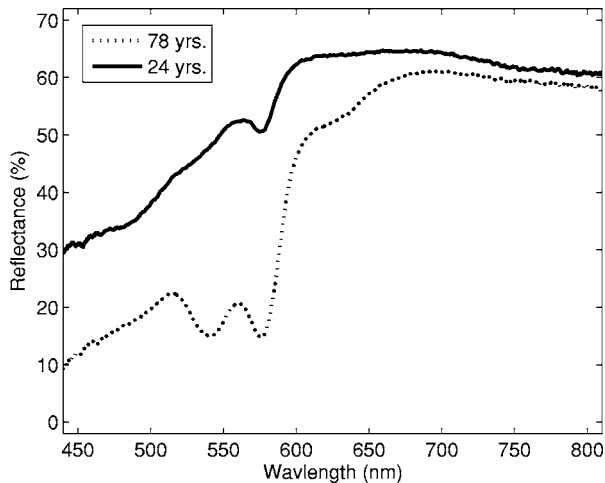
Fig. 16 Variation in reflection spectra in the visible region from normal aorta. In general, the absorption of hemoglobin was stronger the older the person was, and the redder the vessel appeared.

is detected, a high resolution method could be used to make an accurate measurement of the cap thickness.

The aorta is about a factor of 5 larger than the coronary arteries. It must be determined if the method is still sensitive when all sizes are scaled down. Light will penetrate deeper into vessel structures, so a method that only collects light scattered from the plaque will be important.

6 Conclusion

It is shown that NIRS can detect the presence of lipid in plaque in the aorta with good sensitivity. It cannot, however, determine accurately the thickness of the cap. Some other high resolution tomographic method would probably have to be used to determine this. Reflection spectra in the visible region seem to be able to provide some information about lipid content, but yield poorer correlation than the NIR spectral region. Lateral resolution of at least 1 mm is necessary, as the cap thickness can vary throughout the plaque area.

7 Future Studies

It remains to be determined if this detection modality is still possible through a varying layer of blood, or if it is necessary

in some way to press the sensor against the vessel wall. The additional imaging modality that needs to be added must also be determined from viewpoints of resolution, contrast, size, and complexity. It must be determined if the methods are also sensitive in the smaller coronary arteries.

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Table 1 Sensitivities and specificities of the various measurements.

	Circular probe (NIR)	Linear probe (NIR)	Integrating sphere (NIR)	Circular probe (VIS)
Sensitivity	88%	88%	44%	83%
Specificity	94%	81%	91%	79%